The RER for the 17-AAG treatment was 1.64. Previous reports demonstrated that 17-AAG also radiosensitized HCT116 and MCF7 cells.[24,25] We further investigated whether the 17-AAG-mediated radiosensitization was due to enhanced apoptosis. Treatment with either X-ray-irradiation (3 Gy) or 0.5 μM 17-AAG alone induced apoptosis slightly—13.0% and 9.4%, respectively. Importantly, the combination of 17-AAG and X-rays significantly increased the apoptosis (27.5%) (Fig. 5B, right panel).

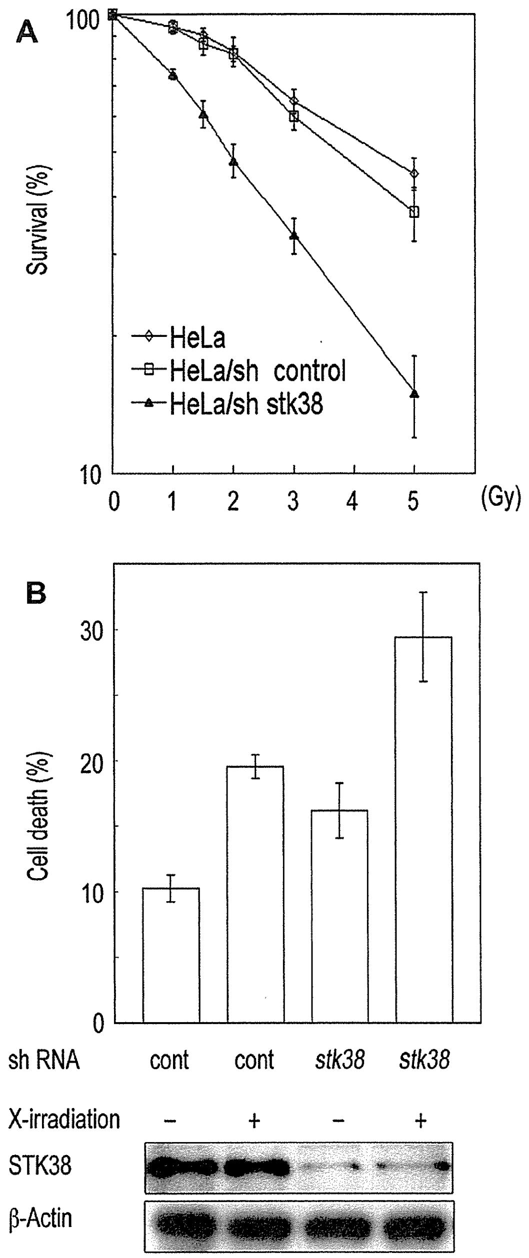

Fig. 6. Serine/threonine kinase 38 (STK38) knockdown enhances X-ray radiosensitivity. (A) HeLa cells were transfected with a non-targeting (control) or *stk38*-specific shRNA expression vector; 24 h later, the cells were placed in culture medium containing 2 μg/ml puromycin and cultured for an additional 48 h. The cells were then either X-ray-irradiated at various doses or left untreated, incubated for an additional 2 h, and assayed for cell survival. (B) HeLa cells were transfected with a non-targeting (control) or *stk38*-specific shRNA expression vector and selected as described above. The shRNA-transfected cells were X-ray-irradiated (3 Gy), and cell death was assessed 48 h later as described in Fig. 5B. STK38 levels were analyzed by Western blot.

3.6. STK38 knockdown enhances X-ray-induced cell death by promoting apoptosis

To clarify the biological significance of *stk38*'s downregulation, we conducted colony-formation assays to determine the effect of STK38 knockdown on cellular radiosensitivity. Transfection with *stk38* shRNA, but not with a control expression vector, specifically knocked down the endogenous STK38 expression in both unirradiated and X-ray-irradiated HeLa cells. When exposed to X-rays, the cell death increased markedly in the *stk38* shRNA-expressing HeLa cells compared to parental HeLa cells or those expressing control shRNA (Fig. 6A). The RER for the knockdown of STK38 at $SF_{0.5}$ was 2.05. Importantly, the combination of *stk38* shRNA and X-irradiation significantly increased the level of apoptosis (29.5%), as indicated by Annexin V staining, compared to that observed in the parental HeLa cells (11.0%) or those expressing control shRNA (19.0%) (Fig. 6B). Thus, STK38 knockdown radiosensitizes cells by promoting apoptosis.

4. Discussion

In this study, we demonstrated that 17-AAG downregulated the *stk38* expression by inhibiting Sp1's DNA-binding activity, and that the reduction of STK38 levels enhanced cellular X-ray radiosensitivity. We initially investigated the effect of 17-AAG on STK38, and found that it decreased both the expression and activity of STK38, in a dose- and time-dependent manner. 17-AAG's disruption of HSP90's binding to client proteins is known to destabilize and degrade those client proteins via the ubiquitin–proteasome pathway.[18,19]

Recently, the mammalian NDR/STK38 homologues LATS1 and LATS2 were identified as HSP90 clients, and 17-AAG was shown to reduce their expression.[26] Thus, we investigated whether STK38 was an HSP90 client. The interaction of STK38 and HSP90 was detected in an overexpression but not an endogenous system (data not shown), and treatment with MG132 or lactacystin did not restore the 17-AAG-mediated reduction in STK38 expression. These observations suggested that while STK38 may interact with HSP90, it is not an HSP90 client protein.

We next found that 17-AAG downregulated *stk38*'s expression. The mechanism regulating *stk38*'s expression has not been clarified. Through 5′-deletion analysis of the *stk38* promoter, we found that the region between −280 and −11 was responsible for the *stk38* promoter activity. This region contained at least two putative Spl-binding sites. Spl is known to bind GC-rich promoter sites, and is considered to be a primary determinant of a promoter's core activity, both by interacting directly with factors in the basal transcriptional machinery and by cooperating with several transcriptional activators.[27,28] In our mutational analysis, the reporter activity decreased slightly with the mutation of one Spl site in the *stk38* promoter, G (−63/−62) T. However, mutations at both Spl-binding sites, G (−73/−72) T and G (−63/−62) T, greatly decreased the promoter activity and Spl's DNA-binding activity, indicating that both sites are necessary for the transcriptional regulation of the *stk38* promoter.

X-ray-irradiation stimulated the STK38 activity without enhancing its expression, probably through phosphorylation-dependent regulation, as we previously reported.[12] Importantly, 17-AAG decreased STK38 expression through reduction of Spl's DNA-binding activity to the *stk38* promoter and reduced STK38 activity in both untreated and X-ray-irradiated cells. A previous study showed that HSP90 interacts with Spl during mitosis and that the inhibition of HSP90 by geldanamycin induces the ubiquitination and degradation of Spl.[29] We confirmed that 17-AAG also induced the degradation of Spl, and that the proteasome inhibitor MG132 rescued the Spl levels. These results suggest that HSP90 protects Spl from ubiquitin-dependent degradation. Moreover, knocking down Spl reduced the STK38 levels, indicating that Spl is necessary for STK38's expression.

Based on the involvement of HSP90 in Spl's stability, our results suggest that 17-AAG downregulates *stk38* expression by decreasing Spl's binding to the *stk38* promoter; this decreased binding is owing to the proteasome-dependent degradation of Spl. On the other hand, JNK1, a member of the JNK family,[30] phosphorylates Spl to protect it from ubiquitination during mitosis, through an interaction among JNK1, HSP90 and Spl.[29] Since MG-132 also increases JNK's activity,[31] JNK may be involved in the prevention by MG-132 of Spl's 17-AAG-induced degradation. JNK is activated by a variety of stimuli, including X-ray-irradiation.[30] However, our results showed that X-irradiation did not stimulate Spl's binding to the *stk38* promoter. This might have been because X-ray-irradiation induces cell-cycle arrest through the activation of checkpoint machinery and transiently inhibits mitosis,[32] leading to a blockade of the JNK1/HSP90/Spl complex formation and of Spl's phosphorylation by JNK during mitosis.

Since the *stk38* promoter activity decreased 43% with the 5′-deletion of the region between −280 and −277, suggesting that another *cis*-acting element exists in this region, we searched for putative transcription factor-binding sites that might support this activity. Computer analysis revealed several potential elements, including Apl- and C/EBP-binding sites. Apl activity is stimulated by oxidative stresses or PMA treatment.[28] However, neither X-ray-irradiation nor PMA treatment stimulated the *stk38* promoter activity, indirectly suggesting that Apl is not involved in regulating the *stk38* promoter. We also found that 17-AAG treatment did not alter the expression of the C/EBP isoform C/EBPβ. This result suggested that C/EBPβ is not responsible for the *stk38* downregulation mediated by 17-AAG, although it might be required for the *stk38* promoter's basal activity.

Preclinical studies have shown that 17-AAG can enhance tumor-cell sensitivity to radiation, while reducing the expressions of radioresistance-associated proteins such as Akt, Raf-1, and Hif-1α.[33,34] Our present study showed that 17-AAG radiosensitized HeLa cells and reduced STK38 expression and that knocking down STK38 significantly enhanced the X-ray radiosensitivity by promoting apoptosis. These results suggest that STK38 is a radioresistance-associated protein. We previously demonstrated that STK38 interacts with and negatively regulates several JNK kinase kinases and that STK38 knockdown enhances stress-induced JNK signaling.[12,13] JNKs are directly linked to cell death.[35] Thus, the 17-AAG-mediated decrease in STK38 or knockdown of *stk38* could activate JNK signaling, leading to an enhancement of X-ray-induced apoptosis. Knocking down *stk38* had a strong radiosensitizing effect, greater than that mediated by 17-AAG, possibly due to differences in the residual STK38 expression levels. The STK38 expression was partially eliminated by 17-AAG treatment, but it was completely eliminated by transfection with an *stk38* shRNA expression vector. Consistent with our results, a recent study demonstrated that silencing STK38 suppresses tumor growth accompanied by increased apoptosis in an *in vivo* mouse xenograft model.[36]

Since Spl is a general transcription factor, it is not particularly suitable for molecular targeting in radiotherapy. On the other hand, STK38 regulates centrosome duplication and protects cells from oxidative stress.[12,37] Moreover, STK38 is upregulated in progressive ductal carcinoma *in situ* and in some melanoma cell lines.[38,39] Thus, radiotherapy and STK38-targeted therapies may have synergistic effects. STK38's substrates and downstream factors still need to be clarified. Our results indicate that STK38 is required to prevent cell death in response to X-irradiation and suggest a new pathway for 17-AAG-mediated radiosensitization via *stk38* downregulation.

Conflict of interest statement

None declared.

Acknowledgements

We thank Dr. Yoshihiro Fujii (Ibaraki Prefectural University of Health Sciences) for helpful discussions and for providing reagents. This work was supported in part by a Grant-in-Aid for Scientific Research (20591491) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to A.E.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2013.06.034.

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Comparison of the bromodeoxyuridine-mediated sensitization effects between low-LET and high-LET ionizing radiation on DNA double-strand breaks

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Received November 13, 2012; Accepted December 17, 2012

DOI: 10.3892/or.2013.2354

Abstract. The incorporation of halogenated pyrmidines such as bromo- and iodo-deoxyuridines (BrdU, IdU) into DNA as thymidine analogs enhances cellular radiosensitivity when high-linear energy transfer (LET) radiation is not used. Although it is known that high-LET ionizing radiation confers fewer biological effects resulting from halogenated pyrimidine incorporation, the exact mechanisms of reduced radiosensitivity with high-LET radiation are not clear. We investigated the radiosensitization effects of halogenated pyrimidines with high-LET radiation using accelerated carbon and iron ions. Cells synchronized into the G_1 phase after unifilar (1 cell cycle) and bifilar (2 cell cycles) substitution with 10 μM BrdU were exposed to various degrees of LET with heavy ions and X-rays. We then carried out a colony formation assay to measure cell survival. The γ-H2AX focus formation assay provided a measure of DNA double-strand break (DSB) formation and repair kinetics. Chromosomal aberration formations for the first post-irradiation metaphase were also scored. For both low-LET X-rays and carbon ions (13 keV/μm), BrdU incorporation led to impaired DNA repair kinetics, a larger initial number of DNA DSBs more frequent chromosomal aberrations at the first post-irradiated metaphase, and increased radiosensitivity for cell lethality. The enhancement ratio was higher after bifilar substitution. In contrast, no such synergistic enhancements were observed after high-LET irradiation with carbon and iron ions (70 and 200 keV/μm, respectively), even after bifilar substitution. Our results suggest that BrdU substitution did not modify the number and quality of DNA DSBs produced by high-LET radiation. The incorporation of halogenated pyrimidines may produce more complex/clustered DNA damage along with radicals formed by low-LET ionizing radiation. In contrast, the severity of damage produced by high-LET radiation may undermine the effects of BrdU and account for the observed minimal radiosensitization effects.

Introduction

Halogenated pyrimidines are well known as classic radiosensitizers for low-linear energy transfer (LET) radiation such as X-rays and γ-rays (1-4). They also have strong sensitization effects for visible and ultraviolet light (5-7). The mechanisms of bromodeoxyuridine (BrdU)-mediated radiosensitization have been explained elsewhere (8-12). Simply put, single-strand break formation from BrdU-mediated radicals results in the formation of lethal DNA double-strand breaks (DSBs). Since sensitization can be partially reduced by adding radical scavengers such as acetone, various reports suggest that BrdU either produces lethal DSBs or fixes potentially lethal damage (PLD) to enhance cell killing (13-16).

High-LET radiation has a strong effect on cell killing when compared to low-LET radiation. Namely, it achieves a higher relative biological effectiveness (RBE) than low-LET radiation (17-20) by producing dense ionization and causing complex, clustered DNA damage (21-24). However, the complex clustered damage produced by high-LET radiation is not fully understood (21). Radiosensitizers are typically less effective when using high-LET radiation when compared with low-LET radiation (25,26).

Reports have indicated that the incorporation of halogenated pyrimidines not only increases the magnitude of radiation-induced DNA damage, but also suppresses DNA damage repair (2,9,16). High-LET radiation produces 'clustered damage', a type of DNA damage which is also difficult to repair (2,9,16). LET-dependent sensitization of halogenated pyrimidines is reported in cellular lethality (25). As LET

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Key words: bromodeoxyuridine, linear energy transfer, double-strand break

increases, increased clustered DNA damage is formed. At LET >100 keV/μm, the RBE declines as LET increases (27,28).

We hypothesized that the mechanism of BrdU-induced hypersensitivity to ionizing radiation is based on the quality of DNA DSBs. We examined the effects of combinations of high-LET heavy ions and unifilar and bifilar BrdU substitution in Chinese hamster ovary (CHO) cells to better understand the BrdU dependency. In this study, we revealed that BrdU substitution followed by low-LET radiation altered DNA damages into more complex damages similar to those observed after high-LET radiation exposure only, while no additional effects on cellular lethality, chromosomal aberrations and DNA DSB formation and repair were observed following high-LET radiation with BrdU.

Materials and methods

Cell lines and culture. Chinese Hamster ovary (CHO10B2) cells (wild-type) were kindly supplied by Dr Joel Bedford of Colorado State University (Fort Collins, CO, USA). Cells were grown in αMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated (56˚C for 30 min) fetal bovine serum (FBS, Sigma, St. Louis, MO, USA) and 1% antibiotics and antimycotics (Invitrogen) in a humidified 5% CO_2 atmosphere at 37˚C. Cell doubling time was ~12 h.

Irradiation and drug treatment. Cells were cultured in a moderately toxic concentration of BrdU (10 μM, Sigma, St. Louis, MO, USA) for our experiments. Log phase cells were cultured in 10 μM BrdU for 10 or 20 h before synchronization to achieve unifilar (>95%) or bifilar (~95%) substitution, respectively. The substitution of BrdU was confirmed by immunocytochemistry against the BrdU antibody (BD, Franklin Lakes, NJ, USA) for unifilar and fluorescence plus Giemsa (FPG) differential staining on metaphase chromosomes for bifilar (29). Cell cycle synchronization was achieved by the mitotic shake-off method (30). Two hours after shake-off, >95% of cells were synchronized in the G_1 phase before they were exposed to ionizing radiation. Cell synchronization was confirmed by flow cytometry. The Titan X-ray irradiator (200 kVp, 20 mA, 0.5-mm Al and 0.5-mm Cu filters; Shimadzu, Japan) yields an X-ray dose of ~1 Gy/min at room temperature. For heavy ion exposure, accelerated ions were irradiated using the Heavy Ion Medical Accelerator in Chiba (HIMAC) at room temperature. Radiation exposure was carried out in a dark environment to prevent cellular toxicity from room light. Dosimetry and beam quality tests for heavy ions were carried out and confirmed by operators of Accelerator Engineering Corp. (Chiba, Japan) (31-34).

Chromosomal aberration assay. To achieve first metaphase arrest, post-irradiated cells were treated with 0.1 μg/ml Colcemid (Sigma) 10-16 h after irradiation. The cells were treated with 75 mM KCl for 15 min at 37˚C. After hypotonic treatment, the cells were fixed with fixative [methanol:acetic acid solution (3:1)] three times and were dropped onto slides. The samples were stained with filtered 10% (v/v) Giemsa solution in Gurr solution (Invitrogen). At least 30 metaphase cells were scored in at least three separate experiments. Chromosomal aberrations were scored as dicentric, fragment, ring, and interstitial and terminal deletion and pooled as total chromosomal aberrations per cell.

Colony formation assay. Cells were trypsinized and plated into P-60 cell culture dishes immediately after the ionizing radiation exposure. Approximately one week later, cells were fixed with 100% ethanol and stained with crystal violet for colony counting. Colonies containing >50 cells were counted as survivors. Plating efficiency was ~75% for the control and 70% for both unifilar and bifilar cells. RBE was calculated from doses required to achieve 10% survival fraction, and the sensitization enhancement ratio (SER) was calculated from the doses required to achieve 37% cell survival.

γ-H2AX formation assay. Synchronized cells were grown on chamber slides for 2 h. After irradiation of 1 Gy and various incubation times (1, 2, or 3 h post-irradiation), the cells were fixed and stained as previously described (35,36). Cellular imaging was accomplished using an Olympus FV300 fluorescence confocal microscope equipped with an Olympus Fluoview three dimensional image analysis system (Olympus, Tokyo, Japan). The foci were scored in at least 50 cells per data point. Three to four independent experiments were carried out.

Statistical analysis. Statistical comparison of mean values was performed using a t-test. Differences with a P-value of <0.05 were considered to indicate a statistically significant result. Error bars indicate standard error of the means. Confidence interval values were calculated by Prism 5™ software (GraphPad, La Jolla, CA, USA).

Results

Comparison of the BrdU substitution-induced radiosensitization effect with X-rays and heavy ions in a colony formation assay. For X-rays and carbon ions with LET of 13 keV/μm, BrdU-incorporated cells were more sensitive to ionizing radiation when compared with the BrdU-negative controls (Fig. 1A and B). Both the initial shoulder and slope of the survival curves were affected by BrdU incorporation. The sensitization effect of BrdU bifilar substituted cells was stronger than unifilar incorporation. In contrast, for higher LET using heavy ions such as carbon ions at LET of 70 keV/μm or iron ions at LET of 200 keV/μm, BrdU substitution did not induce synergistic sensitization with ionizing radiation (Fig. 1C and D). The D_{10} value, the dose which resulted in 10% cell survival and the D_{37}, value, the dose which resulted in 37% cell survival, decreased depending on the level of BrdU incorporation (Table I). For example, the D_{10} value of 5.4 for X-irradiated unlabeled cells was reduced to 3.6 in bifilarly labeled cells, and that of 4.9 for 13 keV/μm LET carbon ion-exposed unlabeled cells was reduced to 3.4 in bifilarly labeled cells. For high-LET radiation, the change in D_{10} value of LET 70 keV/μm carbon ions was from 2.7 to 2.3, and that of LET 200 keV/μm iron ions was from 2.4 to 2.1 for unlabeled to bifilar BrdU substitution. Differences between sets of D_{10} values were statistically significant. On the other hand, for the D_{37} values of unlabeled and bifilar BrdU substitution for high-LET carbon and iron ions, differences between them were regarded as statistically not significant.

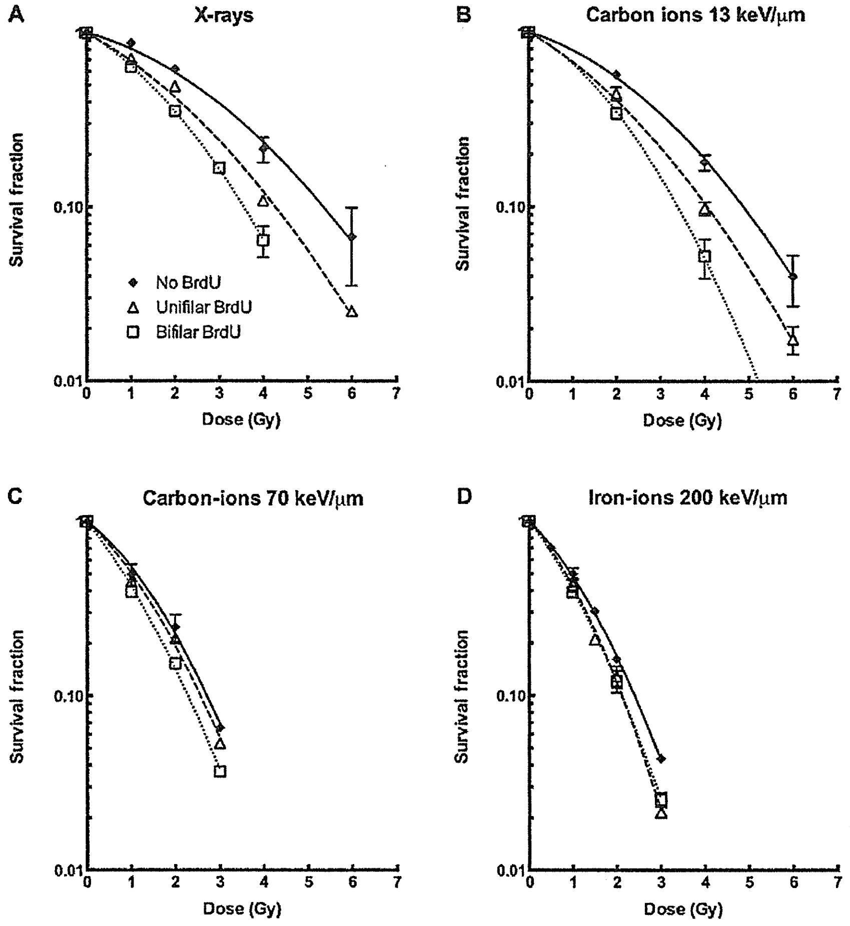

Figure 1. Clonogenic survival curves for different radiation qualities and BrdU incorporation. (A) X-rays, (B) LET 13 keV/μm carbon ions, (C) LET 70 keV/μm carbon ions, (D) LET 200 keV/μm iron ions. ◆, No BrdU substitution; Δ, unifilar substitution; □, bifilar substitution. Three to five independent experiments were carried out. Error bars indicate standard error of the means. Error bars smaller than symbols are not visible. Curves were drawn by GraphPad Prism 5 with linear quadratic regression.

Table I. Relationship between the D_{10} and D_{37} values and BrdU incorporation for different radiation qualities.

Radiation	D_{10}, D_{37}	No BrdU	Unifilar BrdU	Bifilar BrdU
X-ray	D_{10}	5.4 (4.89-5.86)	4.3 (4.08-4.45)	3.6 (3.39-3.72)
	D_{37}	3.1 (2.49-3.58)	2.3 (2.00-2.51)	1.9 (1.68-2.15)
Carbon ions	D_{10}	4.9 (4.46-5.20)	4.1 (3.77-4.28)	3.4 (2.98-3.66)
13 keV/μm	D_{37}	2.9 (2.16-3.33)	2.2 (1.80-2.50)	1.9 (1.48-2.30)
Carbon ions	D_{10}	2.7 (2.47-2.96)	2.6 (2.46-2.68)	2.3 (2.20-2.36)
70 keV/μm	D_{37}	1.5 (1.13-1.74)	1.4 (1.21-1.49)	1.1 (1.02-1.25)
Iron ions	D_{10}	2.4 (2.33-2.49)	2.1 (1.99-2.23)	2.1 (1.97-2.24)
200 keV/μm	D_{37}	1.3 (1.18-1.38)	1.1 (0.95-1.29)	1.2 (0.89-1.25)

D_{10} and D_{37} values were calculated from GraphPad Prism 5. Mean and 99% confidence interval are shown. Experiments were carried out at least three times to obtain the data.

Comparison of BrdU substitution-induced radiosensitization effect with X-rays and heavy ions in a chromosomal aberration assay. As previously shown in the colony formation assay, BrdU-mediated radiosensitization was impaired as LET increased (Fig. 1C and D). To further investigate the mechanism of cell killing, we analyzed first post-irradiation metaphase chromosomes with a chromosomal aberration assay. As predicted from the survival data, no additional

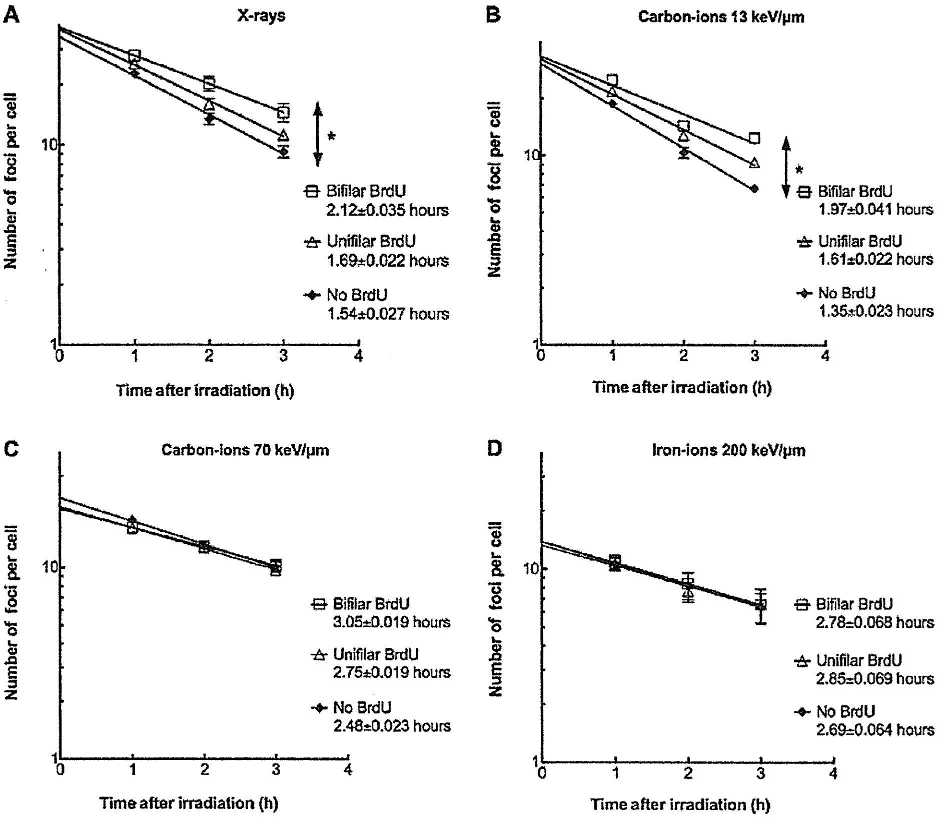

Figure 2. γ-H2AX focus formation assay. (A) X-rays, (B) LET 13 keV/μm carbon ions, (C) LET 70 keV/μm carbon ions, (D) LET 200 keV/μm iron ions. ◆, No BrdU substitution; Δ, unifilar substitution; □, bifilar substitution. Half-life and standard error of the means are shown. Three to four independent experiments were carried out. Error bars indicate standard error of the means. Error bars smaller than symbols are not visible. *Statistical significance ($P<0.05$, t-test) between 0 and 1 cycle and between 0 and 2 cycles of BrdU incorporation.

Table II. Chromosomal aberration assay in first post-irradiation metaphase following irradiation at G1 phase with BrdU substitutions.

Radiation	Dose	No BrdU	Unifilar	Bifilar
No irradiation	0 Gy	0.05±0.03	0.11±0.05	0.15±0.05
X-ray	1 Gy	0.30±0.03	0.48±0.03	0.71±0.04
	2 Gy	0.75±0.05	1.01±0.06	1.33±0.04
Carbon ions 13 keV/μm	1 Gy	0.42±0.05	0.80±0.20	1.00±0.00
	2 Gy	1.06±0.17	1.56±0.24	1.93±0.12
Carbon ions 70 keV/μm	1 Gy	1.26±0.13	1.30±0.25	1.23±0.09
	2 Gy	2.12±0.31	2.54±0.69	2.73±0.37
Iron ions 200 keV/μm	1 Gy	1.34±0.21	1.26±0.26	1.40±0.47
	2 Gy	2.84±0.13	2.68±0.79	2.70±0.64

More than 30 metaphase cells were scored in at least three independent experiments and calculated standard errors of mean.

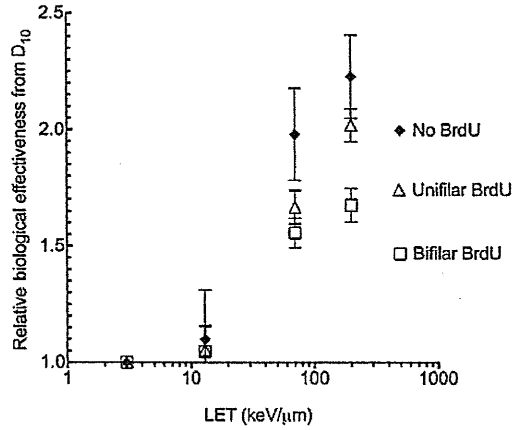

Figure 3. RBE values for different qualities of radiation. RBE values were calculated from the dose to achieve 10% survival. Error bars indicate standard errors of the means.

chromosomal aberrations were observed after BrdU substitution and subsequent high-LET radiation exposure (Table II). After X-ray or LET 13 keV/μm carbon ion exposure, BrdU substitution-mediated radiosensitization was observed at each dose point (1 and 2 Gy) with BrdU incorporation. For instance, bifilar incorporation at 2 Gy increased chromosomal aberrations by 1.71- (from 0.75 to 1.33) and 1.82-fold (from 1.06 to 1.93) for X-rays and LET 13 keV/μm carbon ions, respectively. In contrast, there was almost no radiosensitization at any dose or incorporation time as a result of carbon (70 keV/μm) or iron (200 keV/μm) ions.

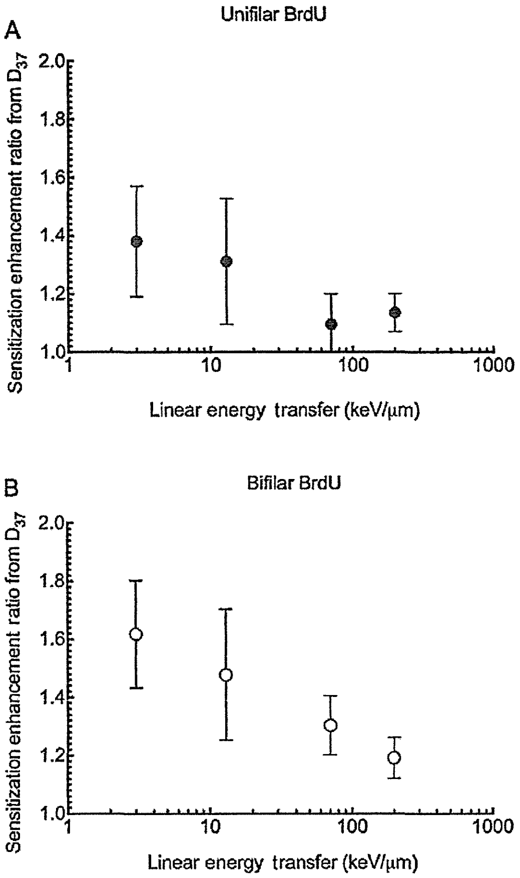

Figure 4. SER values for different qualities of radiation with unifilar (A) or bifilar (B) BrdU substitution. SER values were calculated from the dose to achieve 37% survival, which means the average of one lethal dose per population. Error bars indicate standard errors of the means.

Comparison of BrdU-induced enhancement effect with X-rays and heavy ions in the γ-H2AX formation assay. DNA DSBs result in chromosomal aberrations and cell killing. To investigate the initial amount of DNA damage and DNA repair efficiency following various radiation exposures, we performed a γ-H2AX foci formation assay. One hour after X-ray exposure 22.6 foci per cell for controls, 25.5 foci per cell for unifilar substitution (13% increase), and 27.8 foci per cell for bifilar incorporation (23% increase) were scored. At 3 h after irradiation, the numbers of foci remaining were 9.2 for controls, 12.1 for unifilar incorporation (32% increase), and 14.5 foci for bifilar incorporation (58% increase) (Fig. 2). For LET 13 keV/µm carbon ions, the number of γ-H2AX foci was 18.2 and 6.6 for controls, 21.5 (18% increase) and 9.3 (41% increase) for single BrdU incorporation, and 24.1 (52% increase) and 12.1 (83% increase) for double incorporation at 1 and 3 h, respectively (Fig. 2). Low-LET radiation resulted in an increased number of initial BrdU-induced DNA DSBs and an increased amount of damage remaining in BrdU-incorporated cells. In contrast, high-LET radiation resulted in no significant difference in the number of initial BrdU-induced DNA DSBs or damage remaining in the BrdU-incorporated cells (Fig. 2).

To estimate the effects on repair capacity resulting from BrdU substitution, we calculated half-lives for the reduction of γ-H2AX foci between 1 and 3 h post-irradiation (Fig. 2). Half lives for low-LET exposures increased with the amount of BrdU substitution (from 1.54 to 2.12 h for X-ray bifilar and 1.35 to 1.97 h for bifilar LET 13 keV/µm carbon-ions). In contrast, high-LET exposures (iron-ions in particular) showed no significant difference in γ-H2AX foci half-lives with or without BrdU substitution (from 2.69 to 2.78 h, $P<0.05$).

BrdU substitution effects RBE and SER. In order to assess BrdU substitution effects on radiosensitization, RBE and SER values were obtained. Without BrdU substitution, CHO10B2 cells showed a maximum of an ~2.1 RBE value at LET 200 keV/µm. The LET values were decreased when cells were incorporated with unifilar or bifilar BrdU. The degree of reduction was stronger for bifilar BrdU substitution than unifilar BrdU substitution (Fig. 3). SER values showed that unifilar BrdU substitutions are ~1.4 more effective in cell killing when compared to values without substitution with X-ray exposure, while there was only a 1.1 increase in effectiveness with high LET radiation such as carbon 70 keV/µm and iron ions 200 keV/µm (Fig. 4). The same trend was observed for bifilar substitution. The SER values were decreased from 1.6 to 1.2.

Discussion

In order to investigate the reduced synergistic effects of a combination of high-LET radiation and BrdU incorporation, we compared the damage resulting from low- or high-LET radiation exposure with and without BrdU substitution with several endpoints. We showed that BrdU substitution mediated radiosensitization effects on low-LET photon radiation and particle radiation but similar cellular lethality was observed after high-LET radiation (Fig. 1). Linstadt *et al* (25) proved that the extent of radiosensitization caused by IdU, a halogenated pyrimidine, decreased as the LET increased. In addition, they found very small sensitization enhancements for the distal peak (82 keV/µm) and Bragg peak (183 keV/µm) of a Neon ion beam and no radiosensitization was observed for an extremely high-LET Lanthanum ion beam with 1000 keV/µm (25). Our study was consistent with these results as carbon ions with LET 70 keV/µm and iron ions with LET 200 keV/µm following unifilar or bifilar BrdU incorporation yielded very weak radiosensitization for cellular lethality. Fig. 3 shows the RBE values calculated from D_{10} with BrdU substitution (Table I). Smaller RBE values were noted after high-LET radiation exposure with BrdU substitution. Among the same BrdU substituted cells, high-LET and high RBE advantage was lost. The SER values for high-LET were smaller when cells were incorporated with more BrdU (Fig. 4A and B). When halogenated pyrimidine is used for clinical practice, it is worthy to note that enhancement of cell killing would be smaller using carbon ion radiotherapy than that expected in X-ray radiotherapy. Normal tissues incorporated with BrdU would be severely sensitized following low-LET exposure. Therefore, the dose to patients should be reduced to avoid adverse side effects.

Fig. 2 shows that initial (1 h post-irradiation) DNA damage observed as phosphorylated H2AX foci was increased with BrdU incorporation degrees only for low-LET radiation but not high-LET radiation. Therefore, we assume that initial DNA damages are increased with BrdU substitution for low-LET radiation but not for high-LET radiation. This result was consistent with other reports (4,15). As a result of more initial damage and slower repair, there were additional γ-H2AX foci in BrdU substituted cells after low-LET radiation. γ-H2AX foci are excellent markers for DNA double-strand breaks. H2AX is phosphorylated ~2 mega bases from a DSB site (35,36). We could not exclude the possibility that additional DSBs were produced by BrdU sensitization following high-LET radiation within short range to be recognized as a single focus.

In order to evaluate DNA repair kinetics, we calculated the half-life of γ-H2AX foci from 1 to 3 h after irradiation (Fig. 2). Many DNA DSB repair deficient mutants were found to have slower kinetics of DSB repair and γ-H2AX foci disappearance (37-39). Half-lives of γ-H2AX foci were affected by BrdU substitutions for low-LET radiation, but not for high-LET radiation (Fig. 2). The results coincide with other studies, suggesting that slower repair is one of the possible mechanisms for BrdU-induced radiosensitization (2,13,16,40). In contrast, one study found that halogenated pyrimidines did not effect the repair of PLD for low-LET radiation (X-rays and neon ions with LET 38 keV/μm) and sublethal damage repair (25).

We clearly observed that the combination of BrdU and high-LET radiation does not increase the half-life of γ-H2AX foci disappearance any more than high-LET radiation alone. This suggests that the BrdU substitution did not modify DNA DSBs produced by high-LET radiation or such modifications were naturally formed by high-LET radiation. Multiple publications suggest that high-LET radiation produces dense ionization in their tracks and produce multiple damages near DNA double-strand breaks and form clustered and complex damage (4,12,22,41). These damages are very difficult to repair and it results in high lethality per absorbed physical dose. BrdU-mediated free radicals form lesions such as single-strand breaks, double-strand breaks, and complex double-strand breaks (12). We assumed that BrdU could not contribute any biological response once high-LET produced enough dense ionization on their target. LET >100 keV/μm constitutes an overdose as excess ionizing events do not efficiently produce DSBs (41-43).

These results indicate that there was no detectable difference in the amount of DNA double-strand break formation and no detectable effects for repair kinetics for high-LET radiation with or without BrdU incorporation. These results were directly correlated to no differences in the frequency of chromosomal aberrations in metaphase chromosomes and cellular lethality for high LET radiation with or without BrdU substitution (Fig. 1, Table II).

BrdU and other halogenated pyrimidines appear not to be practical sensitizers to combine with carbon ion radiotherapy due to the severe sensitization to normal tissue and the small sensitization to cancer at higher LET radiation. But LET for the spread out Bragg peak for carbon ion radiotherapy contains a wide range of LET (32,34,44). In this range we would expect some sensitization for tumor control but the SER would not be as high as that for low-LET radiation such as photon and proton radiation.

Acknowledgments

We thank Dr Jamie Bush and Mr. Charles Yurkon of Colorado State University for proofreading our manuscript. This study was partially supported by the Japan Society for the Promotion of Science Grant in Aid (Young Scientists B19710056 to T.K.) and by start-up funds from Colorado State University and Dr Akiko M. Ueno Radiation Biology Research Fund. This study was part of a Research Project with Heavy Ions at the NIRS (National Institute of Radiological Sciences)-HIMAC.

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International Journal of Cancer

Serum interleukin-6 associated with hepatocellular carcinoma risk: A nested case–control study

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Inflammatory markers have been associated with increased risk of several cancers, including colon, lung, breast and liver, but the evidence is inconsistent. We conducted a nested case–control study in the longitudinal cohort of atomic-bomb survivors. The study included 224 hepatocellular carcinoma (HCC) cases and 644 controls individually matched to cases on gender, age, city and time and method of serum storage, and countermatched on radiation dose. We measured C-reactive protein (CRP) and interleukin (IL)-6 using stored sera obtained within 6 years before HCC diagnosis from 188 HCC cases and 605 controls with adequate volumes of donated blood. Analyses with adjustment for hepatitis virus infection, alcohol consumption, smoking habit, body mass index (BMI) and radiation dose showed that relative risk (RR) of HCC [95% confidence interval (CI)] in the highest tertile of CRP levels was 1.94 (0.72–5.51) compared to the lowest tertile ($p = 0.20$). RR of HCC (95% CI) in the highest tertile of IL-6 levels was 5.12 (1.54–20.1) compared to the lowest tertile ($p = 0.007$). Among subjects with BMI > 25.0 kg/m^2, a stronger association was found between a 1-standard deviation (SD) increase in log IL-6 and HCC risk compared to subjects in the middle quintile of BMI (21.3–22.9 kg/m^2), resulting in adjusted RR (95% CI) of 3.09 (1.78–5.81; $p = 0.015$). The results indicate that higher serum levels of IL-6 are associated with increased HCC risk, independently of hepatitis virus infection, lifestyle-related factors and radiation exposure. The association is especially pronounced among subjects with obesity.

Key words: C-reactive protein, interleukin-6, obesity, hepatocellular carcinoma, nested case–control study

Abbreviations: BMI: body mass index; CI: confidence interval; CRP: C-reactive protein; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; IL-6: interleukin-6; RERF: Radiation Effects Research Foundation; RR: relative risk; SD: standard deviation

Conflict of interest: Nothing to report

Grant sponsor: Japanese Ministry of Education, Culture, Sports, Science and Technology; **Grant number:** 20590672; **Grant sponsor:** U.S. Department of Energy (DOE); **Grant number:** DE-HS0000031; **Grant sponsors:** RERF Research Protocols #2-75 and #1-09, Japanese Ministry of Health, Labour and Welfare (MHLW)

DOI: 10.1002/ijc.28337

History: Received 30 Mar 2013; Accepted 31 May 2013; Online 20 Jun 2013

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Int. J. Cancer: **134**, 154–163 (2014) © 2013 UICC

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Chronic infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) are recognized as crucially important risk factors for HCC, whereas an increase of HCC without HBV and HCV infection (non-B, non-C HCC) has been noted recently in Japan.[1,2] Although periodic follow-up with imaging, tumor markers such as alpha-fetoprotein (AFP) and fibrosis markers are recommended, these strategies have not been sufficient for early detection of HCC in chronic liver disease, especially in non-B, non-C liver disease. Therefore, it is necessary to identify biomarkers that may be useful to narrow down a high-risk subgroup for HCC.

A large number of epidemiologic studies have shown that obesity and diabetes mellitus are associated with increased risks of such malignant tumors as colon, prostate and breast, as well as HCC.[3–11] Our earlier study also demonstrated that obesity [body mass index (BMI) > 25.0 kg/m^2] 10 years before HCC diagnosis was significantly associated with increased risk of HCC, independently of HBV and HCV infection, alcohol consumption, smoking habit and radiation exposure.[12] It has been suggested that cell proliferation activity of insulin due to hyperinsulinemia or chronic inflammation may promote

What's new?

According to previous research, alcohol consumption, obesity, and radiation exposure as well as hepatitis virus infection are all independent risk factors for hepatocellular carcinoma (HCC). Inflammatory markers have also been associated with increased risk of liver cancer, but the evidence is inconsistent. In this nested case-control study in the longitudinal cohort of atomic-bomb survivors, which took into account hepatitis virus infection, lifestyle-related factors, and radiation exposure, elevated IL-6 levels were found to be associated with increased risk of HCC. The findings also indicated that association of IL-6 levels with increased risk of HCC is especially pronounced among subjects with obesity.

carcinogenesis by DNA damage, enhancement of cellular proliferation and inhibition of apoptosis.[4,13] In recent years, some studies have suggested that blood levels of inflammatory markers or cytokines also related to insulin resistance—such as C-reactive protein (CRP), interleukin (IL)-6 and tumor necrosis factor (TNF)—may reveal a biological mechanism by which risks of colon, lung and breast cancers increase,[14–17] but other studies have not supported such associations.[18,19]

Several studies have demonstrated that elevated serum levels of IL-6 are associated with increased risk of HCC in female chronic hepatitis C patients,[20] and that the combination of serum levels of IL-6 and alpha-fetoprotein improves sensitivity in diagnosing HCC or predicting future HCC development in chronic hepatitis B patients.[21] A few experimental studies using a mouse model have demonstrated that estrogen-mediated inhibition of IL-6 production by Kupffer cells reduces liver cancer risk in females,[22] and that obesity-promoted HCC development was bound up with elevated production of the tumor-promoting cytokines, such as IL-6 and TNF, which cause hepatic inflammation and activation of the oncogenic transcription factor STAT3.[23] In several other cancers,[24,25] it has been suggested that IL-6 and STAT3 may also contribute toward a general enhancement of cancer risk by high BMI.

With the aim of investigating whether serum levels of CRP and IL-6 are associated with risk of HCC and, if so, whether that risk is independent of HBV and HCV infection, alcohol consumption, smoking habit, BMI and radiation exposure, we conducted a nested case–control study using sera collected from a prospective cohort study of atomic-bomb survivors. We subsequently evaluated whether the association between serum IL-6 levels and HCC risk is modified by alcohol consumption, smoking habit, BMI or radiation dose to the liver using analyses based on subgroups of those factors.

Material and Methods

Cohorts

The Atomic Bomb Casualty Commission (ABCC) and its successor, the Radiation Effects Research Foundation (RERF), established the prospective Adult Health Study cohort in 1958, in which more than 20,000 gender-, age- and city-matched proximal and distal atomic-bomb survivors and persons not present in the cities at the time of bombings have been examined biennially in outpatient clinics in Hiroshima and Nagasaki.

Cases and controls

Incident cancer cases were identified through the Hiroshima Tumor and Tissue Registry and Nagasaki Cancer Registry, confirmed and supplemented by additional cases detected *via* pathological review of related diseases.[26] As described in our previous studies,[3,27] 359 primary HCC cases were diagnosed among 18,660 Adult Health Study participants between 1970 and 2002, who visited our outpatient clinics before their diagnosis. Of these, 229 cases had serum samples obtained within 6 years before HCC diagnosis (average: stored sera obtained 1.2 years before diagnosis). After excluding five cases with inadequate stored serum, 224 cases remained for our previous studies. There were no important differences in characteristics such as alcohol consumption, smoking habit, BMI or radiation dose to the liver (among exposed persons) between HCC cases excluded because of nonavailability of stored serum and those included in our study.

As described in our previous studies,[3,27] 644 controls were selected from the at-risk cohort members matched to the case on gender, age, city and time and method of serum storage, and countermatched on radiation dose in nested case–control fashion.[28] Counter matching (to increase statistical efficiency for studying joint effects of radiation and other factors) was performed using four strata based on whole-body (skin) dose: zero dose (<0.0005 Gy), <0.05 Gy, <0.75 Gy and ≥ 0.75 Gy (nonzero categories correspond roughly to tertiles of skin dose among all eligible exposed cases). At the time of each case diagnosis, one control serum was selected at random from each of the three dose strata not occupied by the case in the cohort risk set.

Laboratory tests

Virological assays of HBV and HCV were performed on 211 cases and 640 controls with sufficient stored sera for these assays as previously described.[29,30] HBV infection (HBV+) status was defined as positive for HBsAg or having a high titer of anti-HBc Ab (positive for anti-HBc Ab of samples diluted 200-fold). HCV infection (HCV+) status was defined as positive for HCV RNA. Non-B, non-C status was defined as negative for HBsAg and not having a high titer of anti-HBc Ab (HBV-) as well as negative for HCV RNA (HCV-).

Serum levels of CRP were measured using an autoanalyzer (Hitachi 7180, Hitachi, Tokyo, Japan) and a high-sensitivity assay kit (Nissui Pharmaceutical, Tokyo, Japan) containing anti-CRP monoclonal antibodies. The detection limit of CRP was 0.08 mg/L. The intra-assay variability was determined by assaying two pooled serum samples (mean CRP level: 0.62 and 1.68 mg/L, respectively) 20 times in a single day, and the respective coefficients of variation (CVs) were 1.12 and 0.95%. The interassay variability was determined by assaying two quality control samples (mean CRP level: 2.14 and 4.71 mg/L, respectively) once a day 12 for days; the respective CVs were 4.1 and 1.2%. Serum levels of IL-6 were measured using the multiplex bead array assay on the Luminex Complete System 200 (Luminex Corp., Austin, TX),[31] with MILLIPLEX^{TM} MAP kits (Millipore, Billerica, MA) according to the manufacturer's instructions. Human serum adipokine panel B (HADK2-61K-B) was used for IL-6. The intra-assay variability was determined by assaying two pooled serum samples with and without including a quality control sample (mean IL-6 level: 4.29 and 144.82 pg/mL, respectively) 15 times in a single day, and the respective CVs were 8.6 and 7.5%. The interassay variability was determined by assaying two quality control samples (mean IL-6 level: 31.02 and 171.62 pg/mL, respectively) once a day for 7 days; the respective CVs were 7.9 and 13.7%.

Radiation dose

Radiation dose to the liver was estimated for each subject according to Dosimetry System DS02.[32] A weighted sum of the gamma dose in gray plus ten times the neutron dose in gray was used.

Alcohol consumption, smoking habit and BMI

Self-administrated questionnaires on lifestyle-related factors were given to Adult Health Study participants in 1965 during attendance at the outpatient clinic and in 1978 by mail survey. Information on alcohol consumption was obtained from the 1965 questionnaire when available, with missing data complemented using the 1978 mail survey. Mean ethanol amounts were calculated as grams per day, as previously described.[33] Information on smoking habit was obtained from the 1965 questionnaire. Subjects were categorized as never, current or former smoker. BMI (kg/m^2) was calculated from height and weight measured in the outpatient clinic of the Adult Health Study. Subjects were classified based on BMI quintiles with cut points of 19.5, 21.2, 22.9 and 25.0. Following the recommendations for Asian people by the WHO, the International Association for the Study of Obesity and the International Obesity Task Force,[34] 21.3–22.9 kg/m^2 was considered as normal, 23.0–25.0 kg/m^2 as overweight and >25.0 kg/m^2 as obese.

Ethical consideration

This study (RERF Research Protocol 1-09) was reviewed and approved by the Research Protocol Review Committee and the Human Investigation Committee of RERF.

Statistical analyses

The nested case–control design is analyzed using a partial likelihood method analogous to that used for cohort follow-up studies,[35] which is in practice the same as the conditional binary data likelihood for matched case–control studies[36] except that the subjects (cases and controls) in the study are not completely independent owing to the possibility of repeated selection. Radiation risk was estimated using an excess relative risk (ERR) model (ERR = RR − 1) to conform to other analyses of the atomic-bomb survivor cohort.[3,27,37] Bias in control doses due to selecting controls using countermatching was corrected using weights as described elsewhere.[28] Risks for all other factors were assessed using a log-linear model. In analyses based on continuous values, CRP and IL-6 were transformed using the natural logarithm. Analyses using CRP or IL-6 groups used tertiles computed among controls. A two-degree-of-freedom heterogeneity test was performed by comparing the deviance of the model with tertiles to that without, using the lowest tertile as the comparison group. We fit log-linear regression models for the effect of a 1-standard deviation (SD) increase in IL-6 and tested for interaction with each of the other risk factors individually using the same heterogeneity test, with degrees of freedom depending on the number of categories of the other risk factor; we report the *p* value for the pairwise test comparing the interaction parameter in the highest to lowest level of each other risk factor. We also assessed various models for log relative risk of HCC with continuous level of IL-6—linear, linear-quadratic and linear spline—using the Akaike information criterion (AIC).[38] Analyses were conducted using Epicure (HIROSOFT International Corp., Seattle, WA).

Results

Characteristics of cases and controls

Table 1 shows characteristics of HCC cases and matched controls. Because of matching, cases and controls were comparable with respect to gender, age, city and time and method of serum storage. Prevalence of HBV and/or HCV infection status in HCC cases is higher than in controls. Compared to the controls, higher proportions of HCC cases had a history of alcohol consumption exceeding 40 g of ethanol per day, were obese (BMI > 25.0 kg/m^2) and were current smokers. Median serum levels of CRP were 0.72 mg/L among HCC cases and 0.59 mg/L among controls. Median serum levels of IL-6 were 4.88 pg/mL among HCC cases and 2.90 pg/mL among controls. HCC cases also received on average higher radiation doses to the liver compared to controls.

Correlations among CRP, IL-6, alcohol, BMI and radiation dose

Table 2 shows Spearman rank-correlation coefficients (*r*) between serum levels of CRP and IL-6, alcohol consumption,

Table 1. Characteristics of HCC cases and controls

Study variables	HCC cases Number with complete data	HCC cases *n* (%)	Controls Number with complete data	Controls *n* (%)
Matched variables				
Age at HCC diagnosis (years)[1]	224	67.6 (10.1)	–	–
Age at serum storage (years)[1]	224	66.4 (10.2)	644	63.7 (9.8)
Gender	224		644	
Male		136 (60.7)		387 (60.1)
Female		88 (39.3)		257 (39.9)
City	224		644	
Hiroshima		155 (69.2)		444 (68.9)
Nagasaki		69 (30.8)		200 (31.1)
Unmatched variables				
Viral etiology	211		640	
HBV–/HCV–		45 (21.3)		579 (90.5)
HBV+ and/or HCV+		166 (78.7)		61 (9.5)
Alcohol consumption (g ethanol/day)	199		577	
None		97 (48.7)		315 (54.6)
0< <40		57 (28.6)		194 (33.6)
≥40		45 (22.6)		68 (11.8)
Smoking habit	199		578	
Never		80 (40.2)		283 (49.0)
Current smoker		107 (53.8)		262 (45.3)
Former smoker		12 (6.0)		33 (5.7)
BMI (kg/m^2) 10 years before diagnosis	210		633	
≤19.5		38 (18.1)		122 (19.3)
19.6–21.2		33 (15.7)		136 (21.5)
21.3–22.9		36 (17.2)		142 (22.4)
23.0–25.0		49 (23.3)		124 (19.6)
>25.0		54 (25.7)		109 (17.2)
Inflammatory markers				
CRP (mg/L), median (IQR)	188	0.72 (0.18, 1.89)	605	0.59 (0.25, 1.52)
IL-6 (pg/mL), median (IQR)	182	4.88 (2.88, 8.77)	589	2.90 (1.53, 5.42)
Radiation dose to the liver (Gy)[1,2]	204	0.46 (0.69)	606	0.34 (0.56)

[1] Mean (SD).
[2] Control values were adjusted for countermatched selection.

BMI 10 years before HCC diagnosis and radiation dose to the liver. Serum levels of CRP were positively correlated with serum levels of IL-6 among both cases ($r = 0.46$) and controls ($r = 0.29$). Serum levels of CRP were modestly correlated with BMI among both cases ($r = 0.15$) and controls ($r = 0.28$), whereas correlations between serum levels of IL-6 and BMI were not significant among either cases ($r = 0.11$) or controls ($r = 0.06$). Neither alcohol consumption nor radiation dose showed any evidence of correlation with either marker.

Risk of HCC according to serum levels of CRP and IL-6

Table 3 shows the association between CRP and HCC risk based on tertiles of serum CRP levels. Analyses with adjustment for HBV and HCV infection, alcohol consumption, smoking habit, BMI 10 years before HCC diagnosis and radiation dose showed that relative risks (RRs) of HCC [95% confidence interval (CI)] in the middle tertile (0.37–0.96 mg/L) and highest tertile (>0.96 mg/L) of CRP levels were 2.11 (0.73–6.54; $p = 0.17$) and 1.94 (0.72–5.51; $p = 0.20$), respectively, compared to

Table 2. Spearman rank-correlation coefficients between CRP, IL-6, alcohol, BMI and radiation dose among HCC cases and controls

	CRP		IL-6	
Variables	Correlation	*p* Value	Correlation	*p* Value
HCC cases				
CRP	–	–	–	–
IL-6	0.46	<0.001	–	–
Alcohol consumption (g ethanol/day)	0.01	0.9	−0.02	0.83
BMI 10 years before diagnosis	0.15	0.049	0.11	0.14
Radiation dose to the liver	−0.09	0.26	−0.08	0.30
Controls				
CRP	–	–	–	–
IL-6	0.29	<0.001	–	–
Alcohol consumption (g ethanol/day)	−0.003	0.94	0.05	0.28
BMI 10 years before diagnosis	0.28	<0.001	0.06	0.13
Radiation dose to the liver	−0.02	0.64	−0.06	0.13

Table 3. Relative risks of HCC by tertile of serum levels of CRP

	Tertile of CRP			*p* Value for heterogeneity
	Low < 0.37 mg/L	Middle 0.37–0.96 mg/L	High > 0.96 mg/L	
No. of cases/controls[1]	**49/120**	**29/98**	**59/109**	
Crude RR (95% CI)	1.00	0.64 (0.36–1.15)	1.16 (0.71–1.88)	0.10
p Value	–	0.14	>0.50	
Adjusted RR (95% CI)[2]	1.00	1.54 (0.62–3.92)	1.90 (0.87–4.36)	0.28
p Value	–	0.36	0.11	
Adjusted RR (95% CI)[3]	1.00	2.11 (0.73–6.54)	1.94 (0.72–5.51)	0.32
p Value	–	0.17	0.20	

[1] Number of subjects for whom information available for all factors included in a log-linear model: 137 HCC cases and 327 controls.
[2] Adjusted for HBV/HCV infection, excluding three HBV+/HCV+ individuals.
[3] Adjusted for HBV/HCV infection, alcohol consumption, smoking habit, BMI 10 years before diagnosis and radiation dose to the liver.

Table 4. Relative risks of HCC by tertile of serum levels of IL-6

	Tertile of IL-6			*p* Value for heterogeneity
	Low < 2.01 pg/mL	Middle 2.01–4.46 pg/mL	High > 4.46 pg/mL	
No. of cases/controls[1]	**13/103**	**48/107**	**71/103**	
Crude RR (95% CI)	1.00	3.78 (1.87–8.26)	6.44 (3.24–14.0)	<0.001
p Value	–	<0.001	<0.001	
Adjusted RR (95% CI)[2]	1.00	2.87 (1.02–8.91)	4.09 (1.46–12.9)	0.025
p Value	–	0.045	0.007	
Adjusted RR (95% CI)[3]	1.00	3.85 (1.16–14.7)	5.12 (1.54–20.1)	0.023
p Value	–	0.027	0.007	

[1] Number of subjects for whom information available for all factors included in a log-linear model: 132 HCC cases and 313 controls.
[2] Adjusted for HBV/HCV infection, excluding three HBV+/HCV+ individuals.
[3] Adjusted for HBV/HCV infection, alcohol consumption, smoking habit, BMI 10 years before diagnosis and radiation dose to the liver.

those in the lowest tertile (<0.37 mg/L; heterogeneity $p = 0.32$).

Table 4 shows the association between IL-6 and HCC risk based on tertiles of IL-6. Analyses with adjustment for HBV and HCV infection, alcohol consumption, smoking habit, BMI 10 years before HCC diagnosis and radiation dose showed that RRs of HCC (95% CI) in the middle tertile (2.01–4.46 pg/mL) and highest tertile (>4.46 mg/L) of IL-6

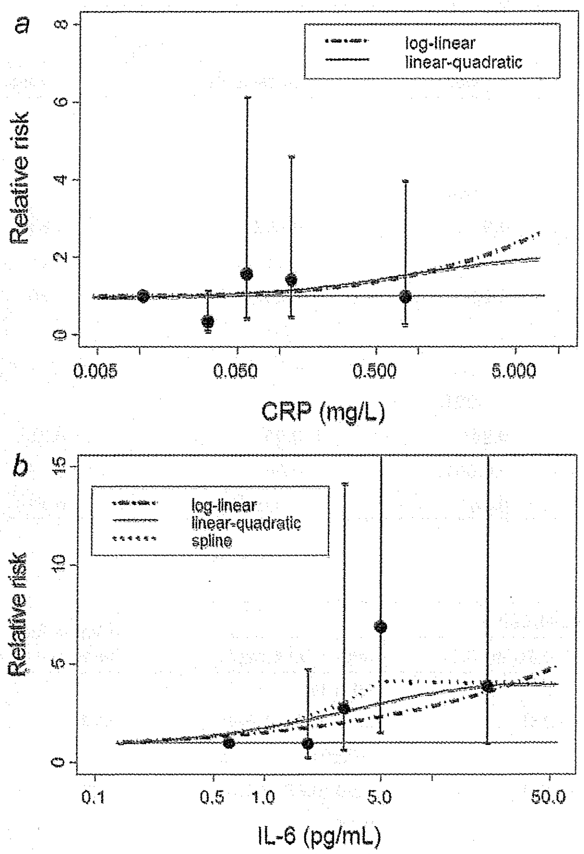

Figure 1. (*a*) Continuous risk of HCC by CRP. RR (95% CI) of HCC with adjustment for alcohol, smoking habit, BMI and radiation dose is plotted according to serum levels of CRP. A test for overall significance of the log-linear curve was not significant (p = 0.23, dashed line). Fit of a linear-quadratic model (solid line) was not as good as the log-linear model according to the AIC model-comparison criterion. (*b*) Continuous risk of HCC by IL-6. RR (95% CI) of HCC with adjustment for alcohol, smoking habit, BMI and radiation dose is plotted according to serum levels of IL-6. A test for overall significance of the log-linear curve was significant (p = 0.015, dashed line). Fits of linear-quadratic (solid line) and linear spline (dotted line) were not as good as the log-linear model according to the AIC model-comparison criterion.

levels were 3.85 (1.16–14.7; p = 0.027) and 5.12 (1.54–20.1; 0.007), respectively, compared to those in the lowest tertile (<2.01 pg/mL; heterogeneity p = 0.023).

Additional analyses were conducted to examine the association between CRP or IL-6 and non-B, non-C HCC risk, although there were relatively few cases with non-B, non-C status (31 cases). Analyses with adjustment for alcohol consumption, smoking habit, BMI 10 years before HCC diagnosis and radiation dose showed that RRs of non-B, non-C HCC (95% CI) in the middle and highest tertiles of CRP were 7.77 (1.13–78.5) and 7.40 (1.26–64.6), respectively, compared to those in the lowest tertile (heterogeneity p = 0.065). RRs of non-B, non-C HCC (95% CI) in the middle and highest tertiles of IL-6 were 56.3 (4.27–2,000) and 98.0 (6.74–4,500), respectively, compared to those in the lowest tertile (heterogeneity $p < 0.001$) after the same adjustment. The wide confidence bounds are presumably due to the small numbers of non-B, non-C HCC cases.

We also examined the possibility of a nonlinear relation between serum levels of CRP or IL-6 and HCC risk. There was no evidence of any systematic relationship between CRP and HCC risk (Fig. 1*a*). The log RR of HCC increased linearly with logarithm of serum IL-6 level after adjustment for alcohol consumption, smoking habit, BMI and radiation dose (p = 0.015, AIC = 132.63; Fig.1*b*). Although HCC risk appears to level off or decline at high values of IL-6 (Fig.1*b*), neither a negative quadratic term (p = 0.17, AIC = 132.73) nor a linear spline (p = 0.10, AIC = 133.95, with best fit obtained using a join point at log IL-6 = 1.6 or IL-6 = 4.95) revealed any statistically significant departure from the log-linear model. Although the appearance of a downturn at high values of IL-6 may be spurious, lack of statistical significance could also be due to the large uncertainty in estimated risk for IL-6 (high upper bound on confidence intervals for IL-6 groups).

Interaction between IL-6 level and gender, lifestyle-related factors or radiation for risks of HCC

Table 5 shows the association between IL-6 and HCC risk by selected subgroups. Stronger association was found between a 1-SD increase in log IL-6 and HCC risk among subjects with BMI of >25.0 kg/m^2 (obese) 10 years before diagnosis than among subjects with BMI of 21.3–22.9 kg/m^2 (normal), resulting in adjusted RR (95% CI) of 3.09 (1.78–5.81; p for interaction = 0.015). However, there was no significant difference in association between IL-6 and HCC risk among females compared to males, among subjects with alcohol consumption of 40 g of ethanol per day compared to never drinkers, among current smokers compared to never smokers or among subjects exposed to $\geq$1.0 Gy radiation compared to subjects exposed to <0.001 Gy radiation.

Additional analyses were conducted to examine the association between IL-6 and non-B, non-C HCC risk by selected subgroups. Similarly, a stronger association was found between a 1-SD increase in log IL-6 and non-B, non-C HCC risk among subjects with BMI of >25.0 kg/m^2 than among subjects with BMI of 21.3–22.9 kg/m^2, resulting in adjusted RR (95% CI) of 5.01 (1.51–34.0; p for interaction = 0.025). The results suggest that elevated serum levels of IL-6 among obese subjects are more strongly associated with increased risks of non-B, non-C HCC as well as overall HCC compared to subjects with normal weight.

Discussion

Our study demonstrated that elevated serum levels of IL-6 are associated with increased risk of HCC, independently of hepatitis virus infection, lifestyle-related factors—such as alcohol consumption, smoking habit and BMI—and radiation

Table 5. Relative risks of HCC associated with a 1-SD increase in log IL-6 level

	RR	95% CI	*p* Value for interaction[1]
All HCC	1.84	1.50, 2.28	
Gender			
Males	1.78	1.36, 2.38	
Females	1.91	1.41, 2.68	>0.5
Alcohol consumption (g ethanol per day)			
None	1.91	1.40, 2.69	
≥40	1.88	1.69, 3.53	>0.5
Smoking habit			
Never	2.09	1.48, 3.07	
Current smoker	1.61	1.19, 2.23	0.28
BMI (kg/m^2) 10 years before diagnosis			
21.3–22.9	1.26	0.80, 1.99	
>25.0	3.09	1.78, 5.81	0.015
Radiation dose to the liver (Gy)			
0≤ <0.001	2.01	1.43, 2.89	
≥1.0	2.50	1.38, 5.10	>0.5
Non-B, non-C HCC	1.62	1.14, 2.39	
Gender			
Males	1.09	0.60, 1.96	
Females	2.13	1.32, 3.84	0.09
Alcohol consumption(g ethanol per day)			
None	1.86	1.09, 3.73	
≥40	2.09	0.57, 11.0	>0.5
Smoking habit			
Never	2.04	1.13, 4.16	
Current smoker	1.35	0.78, 2.39	0.33
BMI (kg/m^2) 10 years before diagnosis			
21.3–22.9	0.84	0.31, 2.02	
>25.0	5.01	1.51, 34.0	0.025
Radiation dose to the liver (Gy)			
0≤ <0.001	1.71	0.89, 3.44	
≥1.0	2.66	1.06, 10.1	0.47

[1] *p* Value for interaction is from the likelihood ratio test for a difference in IL-6 risk between high-risk and reference categories of the other factor, while adjustment was made for main effects and interactions of all categories of the other factor.

exposure. Significant association was observed between elevated serum levels of IL-6 and increased risk of non-B, non-C HCC, whereas the association with elevated serum levels of CRP was only marginally significant. Among subjects with obesity, an even stronger association was observed between elevated serum levels of IL-6 and increased risk of HCC (non-B, non-C HCC as well as all HCC).

Several studies have demonstrated that elevated serum level of CRP is associated with poor prognosis in HCC patients, whereas few cohort studies have shown a significant association between CRP level and HCC risk.[39] In our study, the association between serum level of CRP and HCC risk was not significant, after adjusting for HBV and HCV infection, lifestyle-related factors and radiation dose. However, it has been reported that positive association between CRP level and degree of hepatic steatosis occurs among obese patients with nonalcoholic fatty liver disease,[40] and CRP level is useful not only for distinguishing nonalcoholic steatohepatitis (NASH) from simple nonprogressive fatty liver but also for predicting the severity of liver fibrosis in steatohepatitis cases.[41] In our study, analyses with adjustment for lifestyle-related factors and radiation dose in non-B, non-C subjects showed that the risk of non-B, non-C HCC is significantly higher in the middle or highest tertile of serum CRP levels than in the lowest tertile, and that the risk increases with elevated serum levels of CRP (though only with marginal statistical significance). This result is consistent with published findings that background liver disease of non-B, non-C HCC may be partially caused by NASH or steatohepatitis.[40,41]

Several studies have reported that higher serum IL-6 level precedes the development of HCC in female chronic hepatitis C patients or chronic hepatitis B patients.[20,21] Estrogen-mediated inhibition of IL-6 production by Kupffer cells may explain such gender disparity in HCC development.[22,42–44] An animal study also showed gender-based differences in IL-6 production associated with liver cancers.[22] Previous studies have also demonstrated that serum IL-6 level increases in patients with established HCC.[45] IL-6 is a multifunctional cytokine that plays a prominent role in immune response, cell survival, apoptosis and proliferation.[46] IL-6 produced by inflammatory and stromal cells within the tumor microenvironment binds to gp80 (IL-6 receptor)/gp130 complex, leading to constitutive Janus kinase (JAK) activation and STAT3 phosphorylation, which regulates oncogenic gene expression mediating proliferation and preventing apoptosis.[24] Early studies reported that IL-6 and STAT3 are involved as protumorigenic agents in many cancers, including those of the colon, lung, breast, prostate and ovary, as well as hematological cancers.[46] In our study, the association between serum levels of IL-6 and HCC risk was significant after adjusting for HBV and HCV infection, lifestyle-related factors and radiation dose. Elevated serum levels of IL-6 were associated with increased risk of HCC irrespective of gender. Additionally, analyses with adjustment for lifestyle-related factors and radiation dose in HCC cases and controls of non-B, non-C type showed that non-B, non-C HCC risk is significantly higher in the middle or highest tertile of serum IL-6 levels than in the lowest tertile, and that the risk significantly increases with elevated serum levels of IL-6. These results are consistent with published findings that elevated IL-6 level is associated with the development of type 2 diabetes or insulin resistance,[47] which are considered to be factors contributing to progression in non-B, non-C HCC as well as HCC.

Obesity and diabetes mellitus have recently earned recognition as risk factors for HCC.[4–9] Our previous study[3] also demonstrated that obesity 10 years before HCC diagnosis was an independent risk factor for HCC, and that there was a significant multiplicative interaction in HCC risk between obesity and HCV infection. Obesity contributes to a high rate of visceral fat storage. Increases in production of cytokines such as TNF-α, IL-6, monocyte chemoattractant protein-1 and leptin secreted from adipose tissue and/or macrophages accumulated in such tissues cause hepatic steatosis and oxidative stress through insulin resistance, resulting in the development of HCC. A recent experimental study using a mouse model indicated that obesity promotes HCC development by enhancing production of the tumor-promoting cytokines such as IL-6 and TNF, which cause hepatic inflammation and activation of the oncogenic transcription factor STAT3.[23] In our study, elevated serum levels of IL-6 were significantly associated with increased risk of HCC, especially among subjects with obesity, after adjusting for all other categories of the other risk factor. That trend changed little when the association between IL-6 levels and non-B, non-C HCC risk was examined. Other factors related to HCC risk among obese subjects such as genotype may affect the interaction between IL-6 and obesity, when taking into account the fact that correlations between serum levels of IL-6 and BMI were not significant among HCC cases and controls. Nevertheless, monitoring of IL-6 levels may be crucial to early detection of HCC irrespective of HBV and/or HCV infection, especially for individuals with chronic liver disease or fatty liver disease with obesity.

The strengths of our study include its prospective cohort base with high follow-up rate and nested case–control design, which minimize selection bias. It is difficult and expensive to perform full cohort analyses of serum biomarkers such as IL-6 and CRP, whereas the nested case–control design used here can provide substantial reductions in cost and effort with little loss of statistical efficiency.[48] We also incorporated, in a strict and in-depth manner, hepatitis virus infection status of HCC cases measured before diagnosis (measured at comparable ages among matched controls). Furthermore, we included such potential HCC risk factors as alcohol consumption, smoking habit and BMI in the multivariate analyses, because several studies have demonstrated that inflammatory markers including CRP and IL-6 levels are associated with such lifestyle-related factors.[16,17] However, we cannot completely exclude the possibility of residual confounding.

A limitation of our study is that use of hormones, aspirin and nonsteroidal anti-inflammatory drugs, which are related to CRP levels, could not be adjusted as confounders, because participants have only been asked detailed information on such kinds of medication since 1991. Another is that we used stored sera obtained within 6 years before HCC diagnosis. The reason is that to render primary diagnosis of HBV and/or HCV infection status of cases and controls of serum samples obtained from study participants between 1970 and 2002, *de novo* HCV infection in particular could not be denied outright regarding those obtained between 1970 and 1989. Therefore, the findings of elevated IL-6 levels associated with HCC risk (also measured within 6 years of diagnosis) may include a mixture of precancerous change and defense against tumor formation or growth. It suggests that elevated IL-6 levels may represent not cause but effect for increased risk of HCC, although causality cannot be inferred from our study. However, for early identification and management of HCC, measurement and monitoring of IL-6 levels for individuals with chronic liver disease or fatty liver disease may be meaningful, irrespective of HBV and/or HCV infection.

In conclusion, elevated serum levels of IL-6 were associated with increased risk of HCC, even after adjusting for HBV or HCV infection, alcohol consumption, smoking habit, BMI and radiation dose. Elevated IL-6 levels associated with non-B, non-C HCC risk were also observed, although it was estimated among a relatively small number of non-B, non-C HCC cases. Moreover, elevated serum levels of IL-6 were significantly associated with increased risk of HCC, especially among subjects with obesity. Elevated serum levels of CRP were only marginally associated with increased risk of non-B, non-C HCC, whereas monitoring of CRP and IL-6 levels in combination with tumor markers may be more robust in predicting subsequent HCC among individuals with non-B, non-C liver disease. An in-depth understanding of the mechanisms by which IL-6 levels are associated with increased risk of HCC, independently of hepatitis virus infection, lifestyle-related factors and radiation exposure, should lead to better prevention and therapeutic strategies.

Acknowledgements

The authors thank Dr. Kotaro Ozasa for the provision of the tumor registry information, and all members of the Division of Clinical Laboratories for their excellent assistance. The RERF, Hiroshima and Nagasaki, Japan is a private, nonprofit foundation funded by the Japanese Ministry of Health, Labour and Welfare (MHLW) and the U.S. Department of Energy (DOE), the latter in part through DOE Award DE-HS0000031 to the National Academy of Sciences. This publication was supported by RERF Research Protocols #2-75 and #1-09. The views of the authors do not necessarily reflect those of the two governments.

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