ANTICANCER RESEARCH *41*: 1365-1375 (2021)
doi:10.21873/anticanres.14894

Calorie Restriction Suppresses the Progression of Radiation-Induced Intestinal Tumours in C3B6F1 *Apc*$^{Min/+}$ Mice

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Abstract. *Background/Aim: Progress in cancer treatment and diagnosis has made second cancer after medical radiation exposure a particular concern among childhood cancer survivors. Calorie restriction (CR) is a broadly effective cancer prevention strategy, although its effects on radiation-induced intestinal tumours are unclear. Here we examined the cancer-preventative efficacy of a CR diet at different starting ages on radiation induction of intestinal tumours in mice. Materials and Methods: Male C3B6F1 Apc*$^{Min/+}$ *mice were irradiated with 0 or 2 Gy of X-rays at 2 weeks of age. After an interval of 2, 8 or 18 weeks, mice were fed with a non-CR (95 kcal/week/mouse) or CR (65 kcal/week/mouse) diet. Intestinal tumours were evaluated for number, size distribution and malignancy. Results: CR suppressed the size and progression of both spontaneous and radiation-induced intestinal tumours depending on age at starting of CR. CR diets were effective even administered to adult mice. Conclusion: CR was effective for suppression of tumour progression, which was accelerated by radiation exposure. Use of CR might be a useful cancer-prevention strategy for radiation-induced tumours of the intestinal tract.*

Ionizing radiation has been recognized as a human and rodent carcinogen. Evidence for cancer risk is based on estimates from various sources, including analyses of atomic bomb survivors (1), nuclear power plant accident victims (2), occupational exposure (3), patients undergoing radiation-based medical treatments (4) and animal experiments (5). Children are also estimated to be at a higher risk for developing cancer caused by ionizing radiation than adults (1, 2, 4), due mainly to increased radiosensitivity and a longer lifespan after exposure. Recently, an epidemiological study of atomic bomb survivors has shown that the risk of colorectal cancer (CRC) increased with radiation exposure (6). The colorectum is thus highly susceptible to ionizing radiation exposure.

Medical technologies that use radiation have progressed rapidly for cancer diagnosis and treatments. On the one hand, as a result, the survival of patients after cancer treatment has substantially improved over the past few decades. On the other hand, the increase in medical radiation exposure and the risk of second cancer have become serious problems (7, 8). Second cancer risk is significantly higher when medical radiation exposure takes place during childhood relative to exposure during adulthood (7, 9). Recently, Allodji *et al.* showed the risk of CRC to be significantly increased after radiotherapy of paediatric tumours (10). Hence, it is important to establish a preventative strategy for CRC as second cancer after medical radiation exposure.

Calorie restriction (CR) without incurring malnutrition has been the most well-studied and robust non-genetic, non-pharmacological experimental intervention for extending healthspan and lifespan. Countless studies have confirmed that CR is the only effective intervention for extending the lifespan of many organisms including yeast, worms, flies, rodents and primates (11-17). CR has also been reported to prevent the occurrence of metabolic syndromes such as obesity and to reduce the risk of age-associated diseases such as cancer, diabetes and atherosclerosis in many mammals, including humans (18-23). Although CR is a well-established dietary intervention for cancer prevention in various experimental models, the modifying effects of CR on radiation-associated cancer have not been well described.

The C57BL/6J (B6) *Apc*$^{Min/+}$ mouse is heterozygous for a nonsense mutation at codon 850 in exon 15 of the adenomatous polyposis coli gene (Apc; located on

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Key Words: Calorie restriction, radiation carcinogenesis, cancer prevention.

chromosome 18), which is analogous to APC mutations found in some kindred with familial adenomatous polyposis (24, 25). B6 $Apc^{Min/+}$ mice rarely live past 150 days of age and develop an average of 88 tumours in the small intestinal tract (26). All tumours in B6 $Apc^{Min/+}$ mice are adenomas (ADs), and advanced cancer (*i.e.* invasive cancer) has not been observed. The F1 offspring generated from B6 $Apc^{Min/+}$ mice crossed to C3H mice (*i.e.* B6C3F1 and C3B6F1 mice) show a significant decrease in the number of tumours relative to B6 $Apc^{Min/+}$ mice (26).

We (27) and Yoshida *et al.* (28) reported that CR reduced the risk of hepatocellular carcinoma, lung tumours and myeloid leukaemia in irradiated B6C3F1 and C3H mice, respectively. However, the preventative effects of CR on radiation-induced intestinal tumours have, to date, been unclear. In this study, we examined the influence of radiation exposure on intestinal tumourigenesis in hybrid C3B6F1 $Apc^{Min/+}$ mice. In addition, we also investigated the relevance of the starting age of CR regarding its cancer preventative effects on radiation-induced intestinal tumours.

Materials and Methods

Mice. Male B6 $Apc^{Min/+}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Female C3H/HeJ mice were obtained from Charles River Laboratories (Kanagawa, Japan). Male B6 $Apc^{Min/+}$ and female C3H/HeJ mice were intercrossed to obtain male F1 hybrid C3B6F1 $Apc^{Min/+}$ mice. The Apc genotype was determined by polymerase chain reaction of DNA from a mouse ear punch sample. The F1 mice were housed in a conventional environmentally controlled clean room under the following conditions: 12-h dark/light cycle, temperature of 23±2˚C and 50±10% humidity. The mice were irradiated at 2 weeks of age (see below), weaned at 4 weeks of age and were fed the appropriate diet as indicated below.

Experimental procedure. Male C3B6F1 $Apc^{Min/+}$ mice were randomly divided into eight experimental groups according to whether irradiated or not and to receipt of a non-CR diet (95 kcal/week) or CR diet (65 kcal/week) (Figure 1A). All IR mice were whole-body irradiated with 2 Gy X-rays at 2 weeks of age (IR), when they are most susceptible to radiation (29), and CR was started at 4, 10 or 20 weeks of age (4W, 10W, 20W, respectively). A dose of 2 Gy was chosen to assess the effect of CR on the small intestine because this dose effectively induces small intestinal tumours in B6 $Apc^{Min/+}$ mice (29). X-Ray irradiation (200 kVp, 0.6 Gy/min) was carried out using a Pantak X-ray generator (Pantak, East Haven, CT, USA) as described (30). All mice were weighed weekly during the experimental period. Some mice in the non-CR and IR+non-CR groups were sacrificed at 4, 10 or 20 weeks of age, with all remaining mice sacrificed at 30 weeks of age, after euthanasia under isoflurane anaesthesia for gross and histological examination of small intestinal tumours. All mice were handled according to the principles and procedures outlined in our institutional protocols after authorization by the Institutional Animal Care and Use Committee (authorization number: 12-1029-5).

Experimental diets. The diets consisted of two different calorie-controlled regimens, 65 (CR diet) and 95 (Non-CR diet) kcal/week/mouse. The CR diet contained 32% fewer calories, which were derived from cornstarch carbohydrate, than the non-CR diet (Figure 1B). The CR diet consisted of a reduction in total calorie intake without incurring malnutrition or side-effects such as dysfunction of the liver, kidneys and spleen due to hypogenesis. Details about the food composition of each diet have been reported (28). The diets were fed according to the method previously established in our laboratory (27).

Macroscopic examination. The small intestines were carefully collected, washed with cold saline and opened longitudinally, with the mucosal layer spread out over a piece of filter paper to expose any tumours protruding into the lumen. The tissues were then fixed with 10% phosphate-buffered formalin for 24 h. The number and size of tumours were measured under a stereomicroscope (Leica DFC310 FX digital camera and Leica Application Suite LAS V4.12; Leica Microsystems, Tokyo, Japan). The individual tumours were scored by tumour size as follows: Score 1: <1 mm in diameter; score 2: 1-<2 mm; score 3: 2-<3 mm; score 4: 3-<4 mm; score 5: 4-<5 mm; score 6: ≥5 mm. The 'tumour load' was used to assess the effect of CR and was calculated as described elsewhere (31), with the exception that the sum of scores for all tumours in each mouse was reported.

Histopathological examination. The tumours were embedded in paraffin, sectioned at 4 μm thick and then stained with haematoxylin and eosin for histological analysis. All haematoxylin and eosin specimens were transformed into digital virtual slides using a NanoZoomer-XR slide scanner (Hamamatsu Photonics Corp., Ltd., Hamamatsu, Japan) and stored in a J-SHARE archive (Japan Storehouse of Animal Radiobiology Experiments), which is an animal experiment archiving system constructed in our laboratory (32). The diagnosis of tumours and measurement of tumour diameters were performed with NDP.view2 Plus viewing software (Hamamatsu Photonics Corp., Ltd.), which provides digital histopathological images and direct absolute dimensions such as area and length. The tumours were histologically classified into two diagnostic categories [adenoma (AD) to adenocarcinoma (AC)] according to established criteria (33). The correlation between tumour size and tumour progression, and the ratio of ADs to ACs were analysed statistically as described below.

Biochemical analysis of plasma. Glucose, total cholesterol, triglyceride (TG), non-esterified fatty acid (NEFA), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were measured by using the Milliplex multiplex immunoassay analysis kit (Merck Millipore Darmstadt, Germany). The assay is a novel multiplexed, particle-based, flow-cytometric assay conducted on a Luminex System (Luminex Corp., Austin, TX, USA).

Statistical analysis. The data were analysed using SPSS ver. 24 (IBM Corp., Tokyo, Japan) and GraphPad Prism8.0 (GraphPad Inc., San Diego, CA, USA) software. Statistical differences between two sets of independent data were determined using Student's or Welch's *t*-test based on the results of the *F*-test. A repeated-measures two-way analysis of variance was used to analyse changes in body weight (BW) over time. Histopathologically diagnosed data were analysed using the chi-squared test. Differences with $p<0.05$ were considered statistically significant.

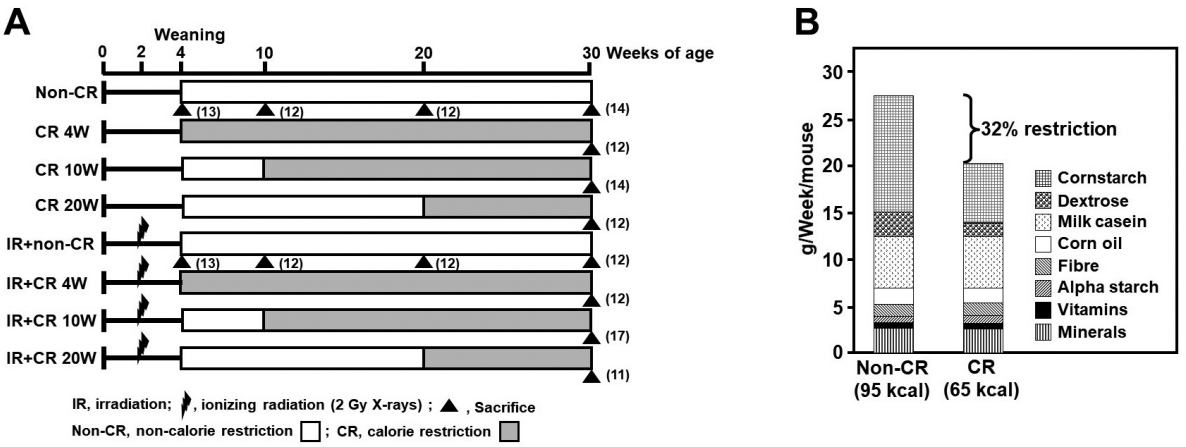

Figure 1. *Experimental design of the study and composition of the non-calorie-restricted and calorie-restricted diets. A: The C3B6F1 Apc$^{Min/+}$ mice were divided into eight groups, treated with or without irradiation (2 Gy of X-rays) at 2 weeks of age and with calorie restriction (CR) (65 kcal/week/mouse) from 4, 10 or 20 weeks of age, or without CR (non-CR, 95 kcal/week/mouse). The numbers in parentheses indicate the number of mice. B: Caloric intake was adjusted by varying the amount of corn starch and dextrose while keeping constant the amounts of other nutrients.*

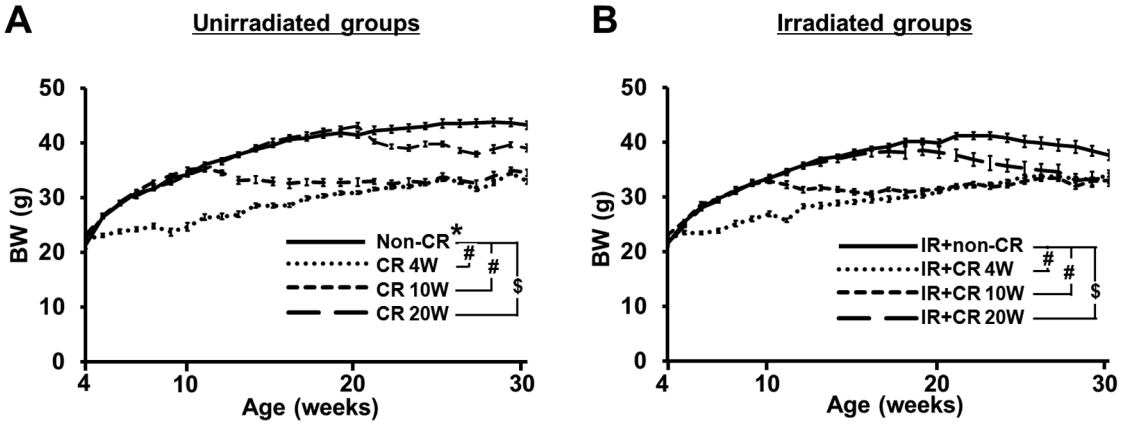

Figure 2. *Time course of body weight (BW) changes in mice treated without (A) or with (B) irradiation (IR) (2 Gy of X-rays) at 2 weeks of age and with calorie restriction (CR) (65 kcal/week/mouse) from 4, 10 or 20 weeks of age, or without CR (non-CR, 95 kcal/week/mouse). CR significantly suppressed weight gain from the first week after the start of the diet. The data shown are the mean±SE. Significantly different at $^{\#}p<0.001$, $^{\$}p<0.05$, and $^{*}p<0.05$ as compared with the IR+non-CR group.*

Results

CR suppressed BW gain and improved lipid metabolism in C3B6F1 Apc$^{Min/+}$ mice. We examined the influence of radiation exposure and CR on BW. Age-dependent changes in the BWs of unirradiated and irradiated mice are shown in Figure 2. The BW in non-CR mice increased gradually with age toward the end of the experimental period (Figure 2A). In contrast, the BW in IR+non-CR mice increased until 23 weeks of age and then slowly decreased with age (Figure 2B). The BW in IR+non-CR mice was significantly lower than that of non-CR mice. In the unirradiated groups (Figure 2A), the BW in all groups that received a CR diet was significantly lower than that in the non-CR group from the first week after the CR diet was fed to the mice until the end of the experimental period. In the irradiated groups (Figure 2B), changes in BW in groups administered the CR diet followed similar patterns to those in the unirradiated groups.

Next, we investigated the effects of radiation exposure on glucose and lipid metabolism. The levels of TG and LDL in 20-week-old irradiated mice showed a 2.7-fold and 3.2-fold (no significant difference) increase as compared with unirradiated mice, respectively (Figure 3C and E). Conversely, the levels of HDL in 20-week-old and 30-week-

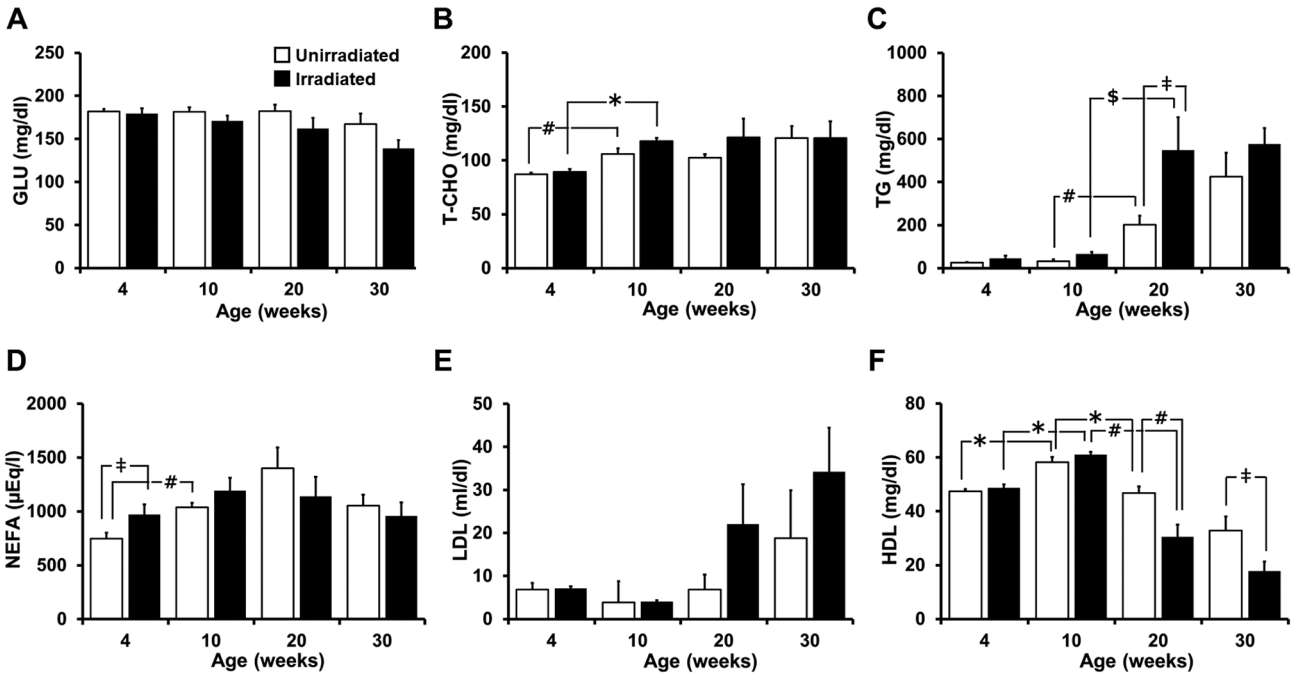

Figure 3. *Influence of time on plasma glucose (GLU) (A), total cholesterol (T-CHO) (B), triglyceride (TG) (C), non-esterified fatty acid (NEFA) (D), low-density lipoprotein (LDL) (E) and high-density lipoprotein (HDL) (F) levels in C3B6F1 Apc$^{Min/+}$ mice fed the non-calorie-restricted diet (95 kcal/week/mouse) treated with or without irradiation (2 Gy of X-rays) at 2 weeks of age. Data shown are the mean±SE. Significantly different at *p<0.001, #p<0.005, $p<0.01 and ‡p<0.05.*

old irradiated mice were significantly lower than those in unirradiated mice (Figure 3F). The levels of glucose, total cholesterol and NEFA did not show dramatic differences between non-CR and IR+non-CR mice (Figure 3A, B and D). These data indicate that radiation exposure accelerated the deterioration of lipid metabolism between 10 and 20 weeks of age.

The effects of CR on biochemical parameters related to glucose and lipid metabolism are shown in Figure 4. CR in both unirradiated and irradiated groups suppressed the levels of TG, NEFA and LDL and conversely increased the HDL levels, as compared with the corresponding non-CR mice. The improvement effects of CR on lipid metabolism were generally stronger when CR was started earlier, although the differences were not significant. Interestingly, even a CR diet started at 20 weeks of age was effective, for which there was a consistent ~200 mg/dl increase in the TG level associated with irradiation. These results indicate that even the late administration of the CR diet was effective in improving lipid metabolic abnormalities caused by irradiation.

Radiation exposure accelerated the increase in the number and size of tumours in C3B6F1 Apc$^{Min/+}$ mice fed the non-CR diet. Gross views of small intestinal tumours in unirradiated and irradiated non-CR mice at 30 weeks of age are shown in Figure 5A and B. Unirradiated mice had a few tumours that were mostly small in size and with a round or oval shape but without constriction at the junction between the tumour and normal mucosa (Figure 5A). Conversely, irradiated mice had numerous tumours that were large and irregularly shaped and showed constriction at the junction between the tumour and normal mucosa (Figure 5B).

Next, we analysed age-dependent alterations in tumour number after radiation exposure (Figure 5C). Only a few tumours were observed in non-CR mice at 4 weeks of age. In the unirradiated mice, the average number of tumours gradually increased in an age-dependent manner up to 30 weeks of age. In contrast, the average number of tumours in the irradiated mice rapidly increased to 2.3-fold of that in the unirradiated mice during the period between 10 and 20 weeks of age; the number at 30 weeks of age was maintained at this high level. The average number of tumours at 20 and 30 weeks of age in the irradiated mice was significantly higher than that of the unirradiated mice.

We also examined the influence of time after radiation exposure on the size distribution of tumours (Figure 5D). The size distribution of tumours at 4 and 10 weeks of age showed similar patterns in both unirradiated and irradiated mice. In irradiated mice at 20 weeks of age, the number of tumours with a diameter of <3 mm was significantly increased as

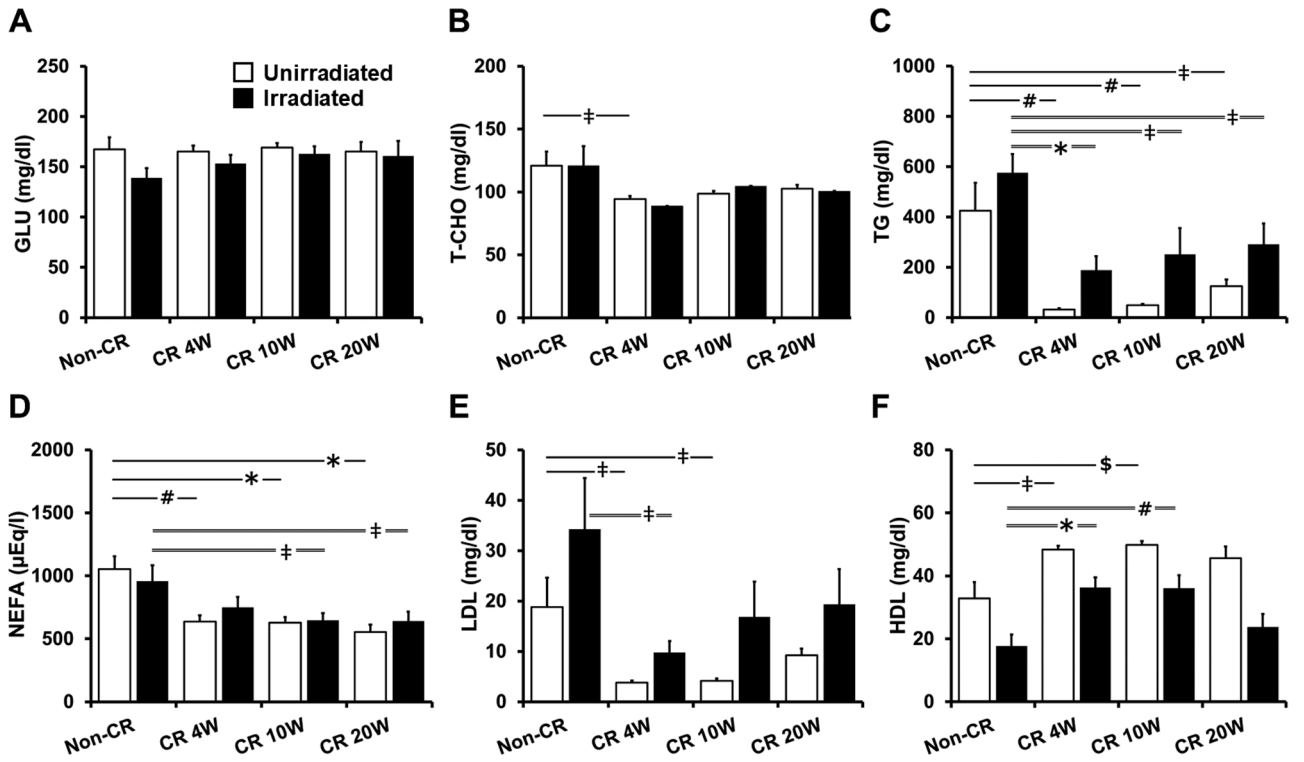

Figure 4. *The effects of diet on plasma glucose (GLU) (A), total cholesterol (T-CHO) (B), triglyceride (TG) (C), non-esterified fatty acid (NEFA) (D), low-density lipoprotein (LDL) (E) and high-density lipoprotein (HDL) (F) levels at 30 weeks of age in C3B6F1 Apc$^{Min/+}$ mice treated with or without irradiation (2 Gy of X-rays) at 2 weeks of age and fed with calorie restriction (CR) (65 kcal/week/mouse) from 4, 10 or 20 weeks of age, or without CR (non-CR, 95 kcal/week/mouse). Data shown are the mean±SE. Significantly different at *p<0.001, $^{\#}$p<0.005, $^{\$}$p<0.01 and ‡p<0.05. W: Weeks.*

compared with those in the unirradiated mice. By the same token, in irradiated mice at 30 weeks of age, the number of tumours with a diameter of 2-<3 mm and of ≥5 mm was significantly increased as compared with the unirradiated mice. As the above results indicate, the C3B6F1 *Apc*$^{Min/+}$ mice underwent an increase in the number and size of tumours after radiation exposure in an age-dependent manner.

CR suppressed the growth of spontaneous and radiation-induced intestinal tumours. We next examined the effects of CR on the number and size of tumours in unirradiated and irradiated C3B6F1 *Apc*$^{Min/+}$ mice. In the unirradiated groups, the CR diet significantly reduced the average number of tumours compared with the non-CR diet when started from 4 or 10 weeks of age (Figure 6A). CR from 4 or 10 weeks of age also significantly reduced the number of tumours with a diameter of ≥2 mm and, conversely, significantly increased the number of tumours with a diameter of <1 mm (Figure 6B). In contrast, the average number of tumours in the irradiated groups did not differ between the CR and non-CR groups (Figure 6A). Therefore, we next analysed the effects of CR on the size distribution of tumours in irradiated mice. CR significantly reduced the number of tumours with a diameter of 2-<3 mm in IR+CR 4W and IR+CR 10W, 4-<5 mm in IR+CR 4W, and ≥5 mm in IR+CR 4W, IR+CR 10W and IR+CR 20W groups. Conversely, it significantly increased the number of tumours with a diameter of <1 mm in IR+CR 4W and IR+CR 10W, and 1-<2 mm in IR+CR 4W and IR+CR 10W groups (Figure 6B). Consistent with the above findings, the average tumour loads of unirradiated and irradiated groups fed the CR diet from 4 or 10 weeks of age were significantly lower than those of the non-CR and IR+non-CR groups, respectively (Figure 6C). These results suggest that the earlier administration of CR diet is useful for suppression of growth of both spontaneous and radiation-induced intestinal tumours.

CR inhibited progression of spontaneous and radiation-induced intestinal tumourigenesis. We further validated the effects of CR on malignant progression from a histopathological viewpoint. The typical histological findings of normal mucosal epithelium and tumours are shown in Figure 7. Tumours were divided into two types, AD (Figure 7B) and AC (Figure 7C), the latter of which exhibited invasion into the proper muscular

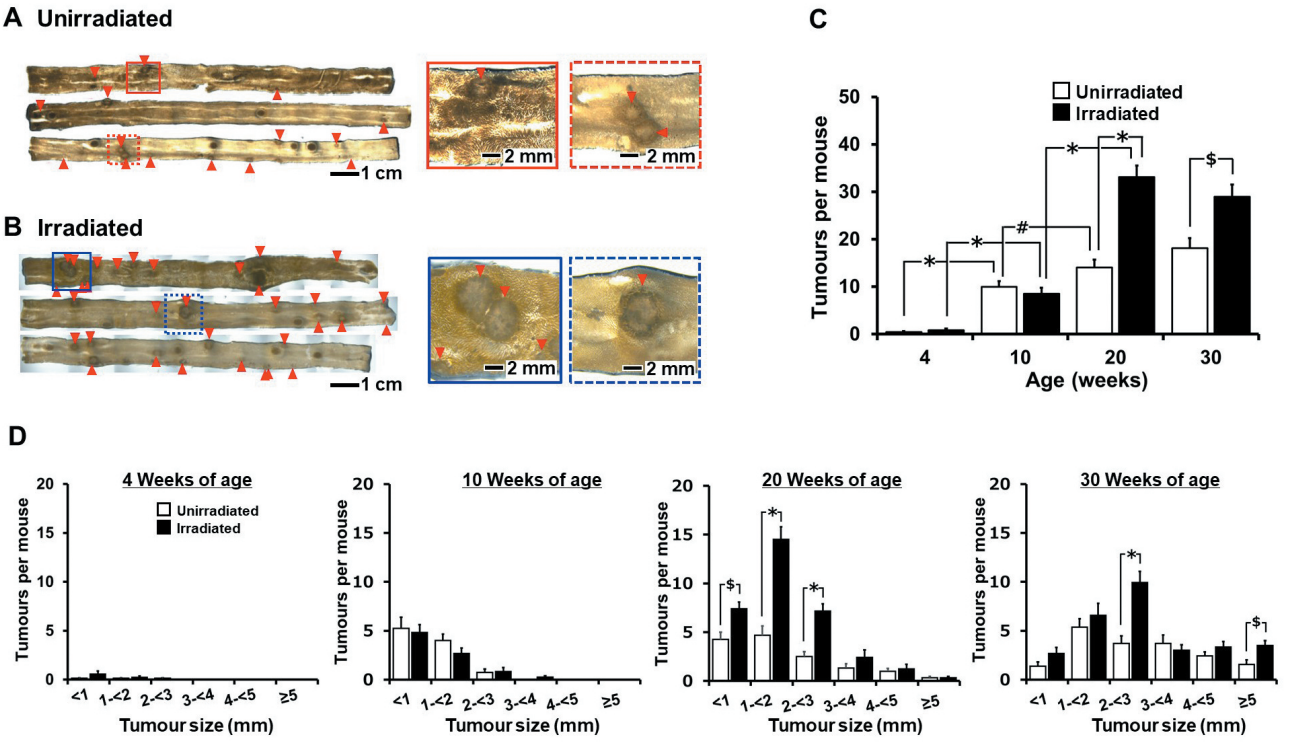

Figure 5. *Influence of time after radiation exposure on the number and size distribution of tumours in C3B6F1 Apc$^{Min/+}$ mice. Mice were irradiated with 0 or 2 Gy of X-rays at 2 weeks of age and fed a non-calorie-restricted diet (95 kcal/week/mouse). Macroscopic views of the small intestine from an unirradiated (A) and irradiated (B) C3B6F1 Apc$^{Min/+}$ mouse. Gross inspection (left) identified tumours of various sizes (red arrowheads) on the mucosal surface of the small intestine; individual tumours in boxed regions are shown at higher magnification (right). C: The average number of tumours per mouse at each age. Radiation exposure increased the number and size of tumours relative to those in mice of the unirradiated group. D: Comparison of tumour size distribution between unirradiated and irradiated mice at each age. Data shown are the mean±SE. Significantly different at *p<0.001, $^{\#}$p<0.005, $^{\$}$p<0.01 and ‡p<0.05.*

layer, as in human CRC. Tumour grading based on histological analysis showed that ACs were significantly larger than ADs in both unirradiated and irradiated groups, regardless of CR (Figure 7D). In addition, radiation exposure significantly increased the proportion of ACs from 31% (non-CR) to 58.5% (IR+non-CR) (Figure 7E).

We next examined the effect of the age at starting CR on tumour progression. The proportions of ACs in the CR 4W and IR+CR 4W groups were significantly lower than those in the non-CR and IR+non-CR groups, respectively (Figure 7E). In addition, CR from 10 and 20 weeks of age also exhibited a tendency in the irradiated groups to suppress the proportion of ACs as compared with the non-CR diet, although this result was not significant (Figure 7E). These results indicated that CR particularly suppressed the malignant progression from AD to AC, which is accelerated by irradiation. Moreover, earlier administration of the CR diet was more effective in suppressing progression of both spontaneous and radiation-induced tumours.

Discussion

In the present study, we described the characteristics of intestinal tumourigenesis and radiation sensitivity in the C3B6F1 *Apc*$^{Min/+}$ mice. We then clarified the modifying effects of CR on both spontaneous and radiation-induced intestinal tumourigenesis.

The C3B6F1 *Apc*$^{Min/+}$ mice developed a smaller number of tumours spontaneously and thus survived for a longer period of time relative to the B6 *Apc*$^{Min/+}$ mice, making it possible to histopathologically observe the process of tumour progression until an advanced, invasive stage. In fact, ~30% of the spontaneous tumours in this model had progressed to invasive cancer by 30 weeks of age, and the histopathology of multistage tumourigenesis closely resembled that of human CRC. In contrast, the widely used B6 *Apc*$^{Min/+}$ mice develop multiple small intestinal ADs by 10-18 weeks of age, and the number of ADs in this mouse model has been used as a convenient indicator of tumourigenesis for studies on cancer prevention (34, 35). However, in the B6 *Apc*$^{Min/+}$ mouse model, the

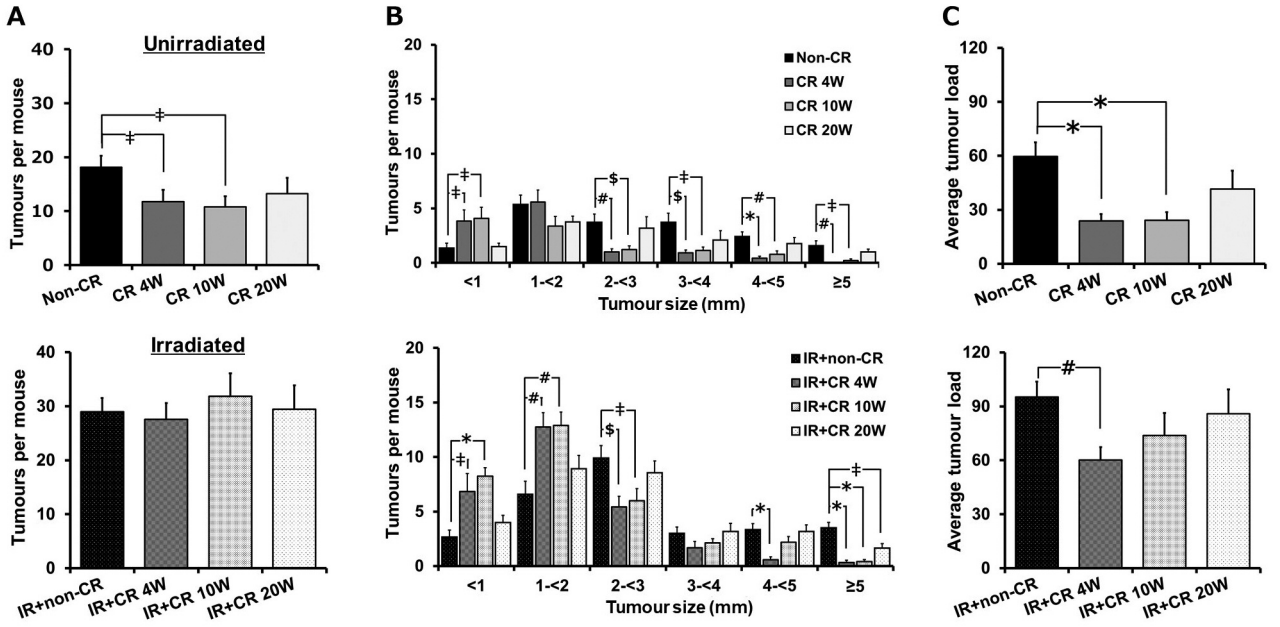

Figure 6. *Effects of diet on the tumour number (A), size distribution of tumours (B), and tumour load (C) in 30-week-old C3B6F1 Apc$^{Min/+}$ mice. Mice were irradiated with 2 Gy of X-rays at 2 weeks of age (lower panel) or not (upper panel) and were fed with calorie restriction (CR) (65 kcal/week/mouse) from 4, 10 or 20 weeks of age, or without CR (non-CR, 95 kcal/week/mouse). Average tumour load was determined by summing all scores corresponding to tumour size for a given animal (see Materials and Methods). Data shown are the mean±SE. Significantly different at *p<0.001, #p<0.005, $p<0.01 and ‡p<0.05. W: Weeks.*

multistage carcinogenesis process cannot be analysed histopathologically due to its short lifespan (~150 days), which is caused by severe anaemia as a result of its heavy tumour burden. Therefore, the C3B6F1 *Apc*$^{Min/+}$ mice used in the present study are considered to be a more suitable model for studies using the histological tumour grade as an indicator. Exposure of the C3B6F1 *Apc*$^{Min/+}$ mice to radiation increased their tumour numbers by 2.3-fold and accelerated the progression of benign tumours to malignancy. Induction of intestinal tumours by radiation exposure has been studied using both B6 and several hybrid *Apc*$^{Min/+}$ mouse strains (29, 36-38). Among them, the study by Okamoto *et al*. (29) is the only one that used an experimental protocol that was similar to ours, including the age at exposure (10 days of age) and an X-ray dose of 2 Gy. They showed that the radiation-related increase in tumour number in the B6 and the (MSM×B6) F1 *Apc*$^{Min/+}$ mice was ~1.4-fold at 16-20 weeks of age and ~2.3-fold at 35-40 weeks of age, respectively (29). In the (MSM×B6) F1 *Apc*$^{Min/+}$ mice, the increase in tumour number was similar to ours but the size distribution and grading of tumours were not shown. In other studies, an increase in tumour number was reported for several hybrid mice with the B6 genetic background, although exposure in those studies occurred most often during adulthood (36-38). Our results thus are the first to show in detail the characteristics of intestinal tumourigenesis after radiation exposure at infancy (2 weeks of age) by using the advantages of C3B6F1 *Apc*$^{Min/+}$ mice. The current study showed that CR suppressed BW gain and improved lipid metabolism in unirradiated and irradiated mice. Our study also indicated that CR prevented the incidence, as well as growth, of tumours in unirradiated mice, whereas the effect of CR was confined to suppression of tumour growth (especially progression to the malignant stage) in irradiated mice. The early administration of the CR diet (starting at 4 weeks of age) was strongly effective in improving lipid metabolism and suppressing spontaneous tumourigenesis, and even adult administration of the CR diet (starting at 10 and 20 weeks of age) was effective, albeit to a lesser extent. Interestingly, even CR starting from 4 weeks of age did not modify the number of tumours after radiation exposure. In the current study, tumour development was initiated by irradiation before the start of CR, and therefore it was considered that CR would not be able to reduce the number of tumours that had already been initiated. Consistently, CR was effective in suppressing tumour growth, and its effect depended on the age at which this dietary regimen was started. Our present study thus first clarifies the relevance of the age at starting of CR on its modifying effects on lipid metabolism and cancer prevention after radiation exposure.

Numerous biological effects of CR have been reported, and proposed mechanisms of its anticancer effects have been

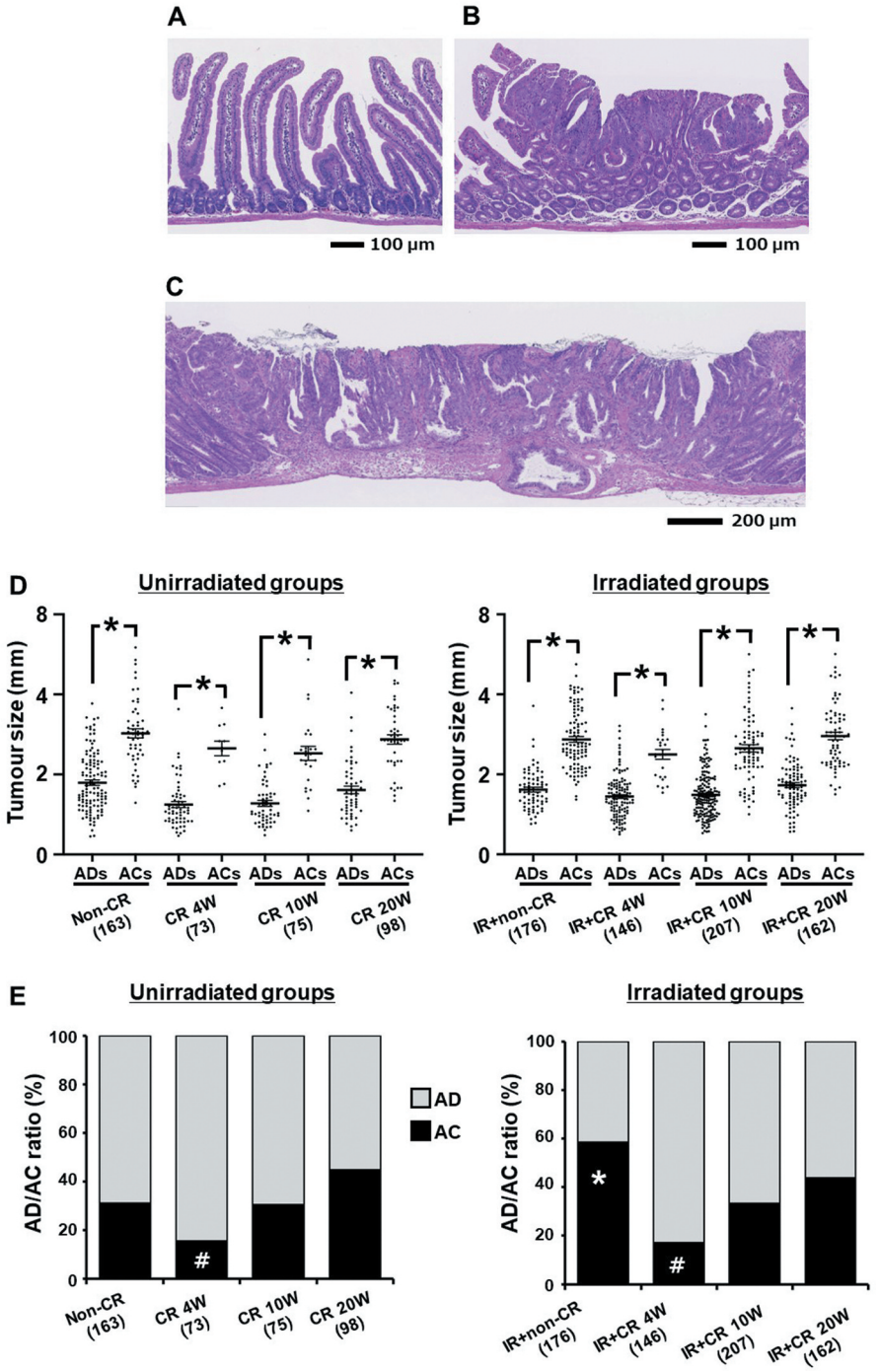

Figure 7. *The effect of diet on tumour progression in 30-week-old C3B6F1 Apc$^{Min/+}$ mice. Mice were irradiated with 2 Gy of X-rays at 2 weeks of age (IR) or not and were fed with calorie restriction (CR) (65 kcal/week/mouse) from 4, 10 or 20 weeks of age, or without CR (non-CR, 95 kcal/week/mouse). Resected intestinal tumours from mice were stained with haematoxylin and eosin, assessed under microscopy and photographed. Representative images are shown. A: Normal histology of small intestinal mucosa in a CR 4W mouse. B: Adenoma found in an unirradiated non-CR mouse localized within the mucosa. C: Adenocarcinoma deeply invading the outer layer of the muscularis propria in an irradiated non-CR mouse. Radiation exposure accelerated malignant alteration of small intestinal tumours. D: Average size of adenomas (ADs) and adenocarcinomas (ACs) in unirradiated and irradiated groups. The average size of ACs was significantly larger than that of ADs in mice with and without CR, with and without irradiation (IR) (*p<0.0001). E: The relative proportion of ADs and ACs in unirradiated and irradiated groups. The proportion of ACs in the IR+non-CR mice was significantly higher as compared with the non-CR mice (*p<0.0001). Conversely, the proportion of ACs in the CR 4W mice was significantly reduced as compared with the non-CR mice, with and without IR ($^{\#}$p<0.001). The numbers in brackets indicate the number of tumours analysed histopathologically.*

reviewed (39). It is of note that the suppressive effect of CR on tumour progression has been ascribed to a decrease in growth factor signalling and inflammation. Antihyperlipidemic drugs have also been shown to reduce the number of ADs in B6 $Apc^{Min/+}$ mice (40, 41). It is therefore plausible that our CR regimen had a similar mechanism. As the preventative mechanism of CR is still unclear regarding radiation-induced intestinal tumours, further studies are warranted to elucidate the particular molecular mechanisms underlying CR suppression of tumour progression for translation into effective cancer prevention strategies in cancer survivors receiving radiotherapy. CR and its mimetics are thus a novel candidate strategy for reduction of second cancer risk after medical radiation exposure.

In conclusion, our study revealed that the C3B6F1 $Apc^{Min/+}$ mice are a useful model for studying the tumourigenic properties and cancer-preventative effects of agents such as radiation and CR using histological tumour grade as a human-relevant indicator. In addition, we showed for the first time that the cancer-preventative effect of CR on radiation-induced intestinal tumours, and even of a later-administered CR diet, suppresses tumour progression, which is accelerated by radiation exposure. CR thus has the potential to serve as an effective regimen for the reduction of second cancer risks after radiotherapy.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

T.M., S.Y., H.Y., M.S., M.K. and S.K. performed the experiments, T.M. performed the histopathological analysis of liver, kidney, spleen and small intestinal tumours; T.M. and H.Y. analysed the data, T.M. and S.K. conceived the study; T.M. and S.K. drafted the article; T.M., S.Y., H.Y., M.S., M.K. and S.K. contributed to discussion and review of the final draft; all Authors approved the final article.

Acknowledgements

The Authors thank Drs. Y. Shang and S. Tani, and Ms. Y. Nishimura, M. Okabe, U. Enzaka and Y. Otsuka for assistance with animal experimentation. We also thank Professor Emeritus N. Yoshimi and Dr. K. Arakaki at the Graduate School of Medicine, University of Ryukyus, Okinawa, Japan for a most useful and helpful discussion, advice and comments.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (project number JP26350955).

References

1 Ozasa K, Shimizu Y, Suyama A, Kasagi F, Soda M, Grant EJ, Sakata R, Sugiyama H and Kodama K: Studies of the mortality of atomic bomb survivors, report 14, 1950-2003: An overview of cancer and noncancer diseases. Radiat Res *177(3)*: 229-243, 2012. PMID: 22171960. DOI: 10.1667/rr2629.1

2 Kazakov VS, Demidchik EP and Astakhova LN: Thyroid cancer after chernobyl. Nature *359(6390)*: 21, 1992. PMID: 1522879. DOI: 10.1038/359021a0

3 Leuraud K, Richardson DB, Cardis E, Daniels RD, Gillies M, O'Hagan JA, Hamra GB, Haylock R, Laurier D, Moissonnier M, Schubauer-Berigan MK, Thierry-Chef I and Kesminiene A: Ionising radiation and risk of death from leukaemia and lymphoma in radiation-monitored workers (INWORKS): An international cohort study. Lancet Haematol *2(7)*: e276-281, 2015. PMID: 26436129. DOI: 10.1016/S2352-3026(15)00094-0

4 Kumar S: Second malignant neoplasms following radiotherapy. Int J Environ Res Public Health *9(12)*: 4744-4759, 2012. PMID: 23249860. DOI: 10.3390/ijerph9124744

5 Morioka T, Miyoshi-Imamura T, Blyth BJ, Kaminishi M, Kokubo T, Nishimura M, Kito S, Tokairin Y, Tani S, Murakami-Murofushi K, Yoshimi N, Shimada Y and Kakinuma S: Ionizing radiation, inflammation, and their interactions in colon carcinogenesis in Mlh1-deficient mice. Cancer Sci *106(3)*: 217-226, 2015. PMID: 25529563. DOI: 10.1111/cas.12591

6 Sugiyama H, Misumi M, Brenner A, Grant EJ, Sakata R, Sadakane A, Utada M, Preston DL, Mabuchi K and Ozasa K: Radiation risk of incident colorectal cancer by anatomical site among atomic bomb survivors: 1958-2009. Int J Cancer *146(3)*: 635-645, 2020. PMID: 30873589. DOI: 10.1002/ijc.32275

7 Einstein AJ, Henzlova MJ and Rajagopalan S: Estimating risk of cancer associated with radiation exposure from 64-slice computed tomography coronary angiography. JAMA *298(3)*: 317-323, 2007. PMID: 17635892. DOI: 10.1001/jama.298.3.317

8 Fazel R, Krumholz HM, Wang Y, Ross JS, Chen J, Ting HH, Shah ND, Nasir K, Einstein AJ and Nallamothu BK: Exposure to low-dose ionizing radiation from medical imaging procedures. N Engl J Med *361(9)*: 849-857, 2009. PMID: 19710483. DOI: 10.1056/NEJMoa0901249

9 Brenner D, Elliston C, Hall E and Berdon W: Estimated risks of radiation-induced fatal cancer from pediatric CT. Am J Roentgenol *176(2)*: 289-296, 2001. PMID: 11159059. DOI: 10.2214/ajr.176.2.1760289

10 Allodji RS, Haddy N, Vu-Bezin G, Dumas A, Fresneau B, Mansouri I, Demoor-Goldschmidt C, El-Fayech C, Pacquement H, Munzer M, Bondiau PY, Berchery D, Oberlin O, Rubino C, Diallo I and de Vathaire F: Risk of subsequent colorectal cancers after a solid tumor in childhood: Effects of radiation therapy and chemotherapy. Pediatr Blood Cancer *66(2)*: e27495, 2019. PMID: 30345604. DOI: 10.1002/pbc.27495

11 Fontana L, Partridge L and Longo VD: Extending healthy life span – from yeast to humans. Science *328(5976)*: 321-326, 2010. PMID: 20395504. DOI: 10.1126/science.1172539

12 Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, Allison DB, Cruzen C, Simmons HA, Kemnitz JW and Weindruch R: Caloric restriction delays disease onset and mortality in rhesus monkeys. Science *325(5937)*: 201-204, 2009. PMID: 19590001. DOI: 10.1126/science.1173635

13 Mair W and Dillin A: Aging and survival: The genetics of life span extension by dietary restriction. Annu Rev Biochem *77*: 727-754, 2008. PMID: 18373439. DOI: 10.1146/annurev.biochem.77.061206.171059

14 Lin SJ, Kaeberlein M, Andalis AA, Sturtz LA, Defossez PA, Culotta VC, Fink GR and Guarente L: Calorie restriction extends saccharomyces cerevisiae lifespan by increasing respiration. Nature *418(6895)*: 344-348, 2002. PMID: 12124627. DOI: 10.1038/nature00829

15 Lakowski B and Hekimi S: The genetics of caloric restriction in caenorhabditis elegans. Proc Natl Acad Sci USA *95(22)*: 13091-13096, 1998. PMID: 9789046. DOI: 10.1073/pnas.95.22.13091

16 Lane MA, Ingram DK and Roth GS: Beyond the rodent model: Calorie restriction in rhesus monkeys. Age *20(1)*: 45-56, 1997. PMID: 23604290. DOI: 10.1007/s11357-997-0004-2

17 McCay CM, Crowell MF and Maynard LA: The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935. Nutrition *5(3)*: 155-171, discussion 172, 1989. PMID: 2520283.

18 Fontana L and Klein S: Aging, adiposity, and calorie restriction. JAMA *297(9)*: 986-994, 2007. PMID: 17341713. DOI: 10.1001/jama.297.9.986

19 Fontana L, Meyer TE, Klein S and Holloszy JO: Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. Proc Natl Acad Sci USA *101(17)*: 6659-6663, 2004. PMID: 15096581. DOI: 10.1073/pnas.0308291101

20 Heilbronn LK, de Jonge L, Frisard MI, DeLany JP, Larson-Meyer DE, Rood J, Nguyen T, Martin CK, Volaufova J, Most MM, Greenway FL, Smith SR, Deutsch WA, Williamson DA, Ravussin E and Pennington CT: Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: A randomized controlled trial. JAMA *295(13)*: 1539-1548, 2006. PMID: 16595757. DOI: 10.1001/jama.295.13.1539

21 Hursting SD, Lavigne JA, Berrigan D, Perkins SN and Barrett JC: Calorie restriction, aging, and cancer prevention: Mechanisms of action and applicability to humans. Annu Rev Med *54:*131-152, 2003. PMID: 12525670. DOI: 10.1146/annurev.med.54.101601.152156

22 Lane MA, Ball SS, Ingram DK, Cutler RG, Engel J, Read V and Roth GS: Diet restriction in rhesus monkeys lowers fasting and glucose-stimulated glucoregulatory end points. Am J Physiol *268(5 Pt 1)*: E941-948, 1995. PMID: 7762649. DOI: 10.1152/ajpendo.1995.268.5.E941

23 Longo VD and Fontana L: Calorie restriction and cancer prevention: Metabolic and molecular mechanisms. Trends Pharmacol Sci *31(2)*: 89-98, 2010. PMID: 20097433. DOI: 10.1016/j.tips.2009.11.004

24 Miyoshi Y, Ando H, Nagase H, Nishisho I, Horii A, Miki Y, Mori T, Utsunomiya J, Baba S, Petersen G and *et al*.: Germ-line mutations of the apc gene in 53 familial adenomatous polyposis patients. Proc Natl Acad Sci USA *89(10)*: 4452-4456, 1992. PMID: 1316610. DOI: 10.1073/pnas.89.10.4452

25 Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, Gould KA and Dove WF: Multiple intestinal neoplasia caused by a mutation in the murine homolog of the apc gene. Science *256(5057)*: 668-670, 1992. PMID: 1350108. DOI: 10.1126/science.1350108

26 Koratkar R, Silverman KA, Pequignot E, Hauck WW, Buchberg AM and Siracusa LD: Analysis of reciprocal congenic lines reveals the C3H/HeJ genome to be highly resistant to apcmin intestinal tumorigenesis. Genomics *84(5)*: 844-852, 2004. PMID: 15475263. DOI: 10.1016/j.ygeno.2004.07.010

27 Shang Y, Kakinuma S, Yamauchi K, Morioka T, Kokubo T, Tani S, Takabatake T, Kataoka Y and Shimada Y: Cancer prevention by adult-onset calorie restriction after infant exposure to ionizing radiation in B6C3F1 male mice. Int J Cancer *135(5)*: 1038-1047, 2014. PMID: 24482070. DOI: 10.1002/ijc.28751

28 Yoshida K, Inoue T, Nojima K, Hirabayashi Y and Sado T: Calorie restriction reduces the incidence of myeloid leukemia induced by a single whole-body radiation in C3H/He mice. Proc Natl Acad Sci USA *94(6)*: 2615-2619, 1997. PMID: 9122244. DOI: 10.1073/pnas.94.6.2615

29 Okamoto M and Yonekawa H: Intestinal tumorigenesis in min mice is enhanced by x-irradiation in an age-dependent manner. J Radiat Res *46(1)*: 83-91, 2005. PMID: 15802863. DOI: 10.1269/jrr.46.83

30 Tokairin Y, Kakinuma S, Arai M, Nishimura M, Okamoto M, Ito E, Akashi M, Miki Y, Kawano T, Iwai T and Shimada Y: Accelerated growth of intestinal tumours after radiation exposure in *Mlh1*-knockout mice: Evaluation of the late effect of radiation on a mouse model of HNPCC. Int J Exp Pathol *87(2)*: 89-99, 2006. PMID: 16623753. DOI: 10.1111/j.0959-9673.2006.00464.x

31 Neufert C, Becker C and Neurath MF: An inducible mouse model of colon carcinogenesis for the analysis of sporadic and inflammation-driven tumor progression. Nat Protoc *2(8)*: 1998-2004, 2007. PMID: 17703211. DOI: 10.1038/nprot.2007.279

32 Morioka T, Blyth BJ, Imaoka T, Nishimura M, Takeshita H, Shimomura T, Ohtake J, Ishida A, Schofield P, Grosche B, Kulka U, Shimada Y, Yamada Y and Kakinuma S: Establishing the japan-store house of animal radiobiology experiments (J-SHARE), a large-scale necropsy and histopathology archive providing international access to important radiobiology data. Int J Radiat Biol *95(10)*: 1372-1377, 2019. PMID: 31145030. DOI: 10.1080/09553002.2019.1625458

33 Boivin GP, Washington K, Yang K, Ward JM, Pretlow TP, Russell R, Besselsen DG, Godfrey VL, Doetschman T, Dove WF, Pitot HC, Halberg RB, Itzkowitz SH, Groden J and Coffey RJ: Pathology of mouse models of intestinal cancer: Consensus report and recommendations. Gastroenterology *124(3)*: 762-777, 2003. PMID: 12612914. DOI: 10.1053/gast.2003.50094

34 Asadi K, Ferguson LR, Philpott M and Karunasinghe N: Cancer-preventive properties of an anthocyanin-enriched sweet potato in the *Apc*^{MIN} mouse model. J Cancer Prev *22(3)*: 135-146, 2017. PMID: 29018778. DOI: 10.15430/JCP.2017.22.3.135

35 Khan I, Huang G, Li XA, Liao W, Leong WK, Xia W, Bian X, Wu J and Hsiao WLW: Mushroom polysaccharides and jiaogulan saponins exert cancer preventive effects by shaping the gut microbiota and microenvironment in *Apc*$^{Min/+}$ mice. Pharmacol Res *148:* 104448, 2019. PMID: 31499195. DOI: 10.1016/j.phrs.2019.104448

36 Datta K, Suman S, Kallakury BV and Fornace AJ, Jr.: Heavy ion radiation exposure triggered higher intestinal tumor frequency and greater beta-catenin activation than gamma radiation in *Apc*$^{Min/+}$ mice. PLoS One *8(3)*: e59295, 2013. PMID: 23555653. DOI: 10.1371/journal.pone.0059295

37 Nakayama T, Yamazumi K, Uemura T, Yoshizaki A, Yakata Y, Matsuu-Matsuyama M, Shichijo K and Sekine I: X Radiation up-regulates the occurrence and the multiplicity of invasive carcinomas in the intestinal tract of *Apc*$^{Min/+}$ mice. Radiat Res *168(4)*: 433-439, 2007. PMID: 17903035. DOI: 10.1667/RR0869.1

38 van der Houven van Oordt CW, Smits R, Schouten TG, Houwing-Duistermaat JJ, Williamson SL, Luz A, Meera Khan

P, van der Eb AJ, Breuer ML and Fodde R: The genetic background modifies the spontaneous and x-ray-induced tumor spectrum in the *Apc*1638N mouse model. Genes Chromosomes Cancer *24(3)*: 191-198, 1999. PMID: 10451698. DOI: 10.1002/(sici)1098-2264(199903)24:3<191::aid-gcc3>3.0.co,2-l

39 Hursting SD, Dunlap SM, Ford NA, Hursting MJ and Lashinger LM: Calorie restriction and cancer prevention: A mechanistic perspective. Cancer Metab *1(1)*: 10, 2013. PMID: 24280167. DOI: 10.1186/2049-3002-1-10

40 Chang WL, Jackson C, Riel S, Cooper HS, Devarajan K, Hensley HH, Zhou Y, Vanderveer LA, Nguyen MT and Clapper ML: Differential preventive activity of sulindac and atorvastatin in $Apc^{+/Min\text{-}FCCC}$ mice with or without colorectal adenomas. Gut *67(7)*: 1290-1298, 2018. PMID: 29122850. DOI: 10.1136/gutjnl-2017-313942

41 Swamy MV, Patlolla JM, Steele VE, Kopelovich L, Reddy BS and Rao CV: Chemoprevention of familial adenomatous polyposis by low doses of atorvastatin and celecoxib given individually and in combination to *Apc* Min mice. Cancer Res *66(14)*: 7370-7377, 2006. PMID: 16849589. DOI: 10.1158/0008-5472.CAN-05-4619

Received December 28, 2020
Revised January 22, 2021
Accepted January 25, 2021