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Methylation changes of H_{19} gene in sperms of X-irradiated mouse and maintenance in offspring

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Abstract

The nature of imprinting is just differential methylation of imprinted genes. Unlike the non-imprinted genes, the methylation pattern of imprinted genes established during the period of gametogenesis remains unchangeable after fertilization and during embryo development. It implies that gametogenesis is the key stage for methylation pattern of imprinted genes. The imprinting interfered by exogenous factors during this stage could be inherited to offspring and cause genetic effect. Now many studies have proved that ionizing irradiation could disturb DNA methylation. Here we choose BALB/c mice as a research model and X-ray as interfering source to further clarify it. We discovered that the whole-body irradiation of X-ray to male BALB/c mice could influence the methylation pattern of H_{19} gene in sperms, which resulted in some cytosines of partial CpG islands in the imprinting control region could not transform to methylated cytosines. Furthermore, by copulating the interfered male mice with normal female, we analyzed the promoter methylation pattern of H_{19} in offspring fetal liver and compared the same to the pattern of male parent in sperms. We found that the majority of methylation changes in offspring liver were related to the ones in their parent sperms. Our data proved that the changes of the H_{19} gene methylation pattern interfered by X-ray irradiation could be transmitted and maintained in the first-generation offspring.

Keywords: Ionizing radiation; DNA methylation; Gene imprinting; Spermatogenesis; H19

Ionizing radiation is a well-documented potent mutagen and carcinogen that may result in a series of genetic and epigenetic effects [1–5]. One important epigenetic mechanism is genome methylation, particularly the methylation of the CpG islands, which are frequently found in the promoter regions of genes [6]. DNA methylation modification has a lot of important biological functions, and it is also the mechanism of genome imprinting [7]. Genome imprinting is the phenomenon that imprinted genes conserve certain characteristics of its gametic genome, differentially and alternatively expresses, and is closely related to fetal development.

The imprinting control region (ICR), which exists in imprinted genes, can cause parents' differential gene expression through the changes of methylation patterns in the sites of CpG islands. There are differences of gene methylation in this region between the parents' sperms and eggs, therefore, this region is also called differentially methylated region, DMR. The allele's expression will be closed when the cytosine in CpG islands is converted into methylated cytosine. However, the regulation sequence of the other allele is not methylated, and its histone is acetylated, therefore the mRNA could be transcribed with the action of the transcriptional complex [8].

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In mammalian life cycles, the original methylation patterns of imprinted genes were erased in primordial germ cells; new patterns are established in gametogenesis. For the non-imprinting genes, methylations were eliminated subsequently after the stage of gametogenesis and reestablished in blastura. However, the methylation patterns of imprinted genes established in gametogenesis were unaltered regardless of subsequent demethylation and remethylation, and maintained during fertilization and subsequent embryo development [9]. Therefore, the gametogenesis is the key stage for the formation of imprinted genes. If imprinting is interfered by exogenous factors during this stage, it could cause genetic effect and inherited to offspring as well. Since the nature of imprinting is the formation of differential methylation of imprinted genes, the results of interference to imprinting were the changes of methylation patterns in ICR. Researches have showed that ionizing radiation could induce an alternation of the methylation patterns [10–12]. However, there is no study indicating that ionizing radiation could interfere with the formation of imprinting during gametogenesis, which is necessary to be clarified.

H_{19} is expressed from the hypomethylated maternal allele and repressed on the hypermethylated paternal allele in mice. The paternal-specific methylation of the murine H_{19} locus covers approximately 7 kb, including 4 kb of upstream sequence and the transcription unit [13]. The DMR containing CpG islands is methylated in spermatozoa. The imprinting occurs during spermatogenesis, and all CpG islands were methylated in the stage of mature sperm [14–16]. So H_{19} is a promising target for analyzing gene imprinting during spermatogenesis.

In the present study, to explore whether the radiation could interfere with the formation of imprinting during spermatogenesis, male BALB/c mice were subjected to whole-body irradiation of X-ray and then the methylation patterns of H_{19} DMR in the irradiated mice sperms were examined. In addition, we copulated the radiation-treated male mice with normal female mice to see whether the imprinting alternation induced by radiation could be inherited to offspring. Our results indicated that the methylation patterns of H_{19} gene DMR had been changed in the ionizing radiation-treated mice sperms and could be inherited to the next generation.

Materials and methods

Animal experiments. Eight male BALB/c mice, 7–8 weeks old, were bought from Shanghai Center for Experimental Animal, Chinese Academy of Science, and fell randomly into experimental and control groups. There were four mice in each group. The experimental animals were subjected to whole-body irradiation of total 1.3 Gy X-rays applied as 0.1 Gy per day for 13 days, which were emitted by Siemens KD-2 X-ray accelerator. After 19 days of breeding, these male experimental animals were copulated with maiden female mice (male/female = 1:2). If there was vagina blot in female mouse, we make sure that the copulation was successful, and marked it as 0.5 day of pregnancy. Male mice were killed and their sperms were taken from the tail of epididymis. The female pregnant mice were bred continuously and killed on the 14.5 day of pregnancy. Four pregnant mice bore 29 fetal mice and each pregnant mouse averagely bore 7–8 fetal mice. Two fetal mice were randomly selected from each pregnant mouse, therefore, liver tissues from total eight fetal mice were taken, and DNA was isolated. The mice of control group received the same treatments except for X-ray radiation. The experiment time was according to the cycle of mouse spermatogenesis, there are 35 days from gonad stem cells to mature sperms, of which the stage of spermiogonium lasts 8 days, meiosis lasts 13 days, sperm formation needs 14 days, and it also takes 5 days for sperms to reach the tail of epididymis and ejaculate [17]. The sperms were not at the same spermatogenesis cycle, however, the analysis of methylation patterns of CpG islands in H_{19} ICR of sperm DNA could reflect the interference of X-ray to imprinting process.

DNA isolation and sodium sulfite treatment. DNA from sperms and liver tissues was isolated with DNA easy kit (Qiagen) according to the manufacturer's protocol and finally dissolved in 25–50 μl TE. The DNA concentration and degree of purity were tested with a Biophotometer (Eppendorf). Using CpGenome DNA Modification Kit (Chemicon), DNA was treated with sodium sulfite according to the manufacturer's protocol, and 2 μl of modification reagent IV was added because of the low concentrations of these sperms. The treated DNA samples were finally dissolved in 15–25 μl TE and stored at −80 °C.

PCR amplification, cloning, and sequencing. The primers for amplifying the CpG islands of mice H_{19} MDR were referred to Lucifero et al. [16] and synthesized by the Shengneng Bicolor company. The primers of the first ring reaction of the nested PCR were as follows: outside forward—5′-gagtatttaggaggtataagaatt-3′, outside reverse—5′-atc aaaaactaacataaaccct-3′. The primers of the second ring of the nested RT-PCR were as follows: inside forward—5′-gtaaggagattatgtttatttttgg-3′, and inside reverse—5′-cctcattaatcccataactat-3′. According to the protocol of the Faststart kit supplied by Roche Company, the first ring of 50 μl PCR system contains 8 μl of sodium sulfite-treated DNA, 5 μl of 10× PCR buffer, 10 μl of 5× GC-RICH solution, 2 μl (200 μM) dNTPs, 0.5 μM of each primer, and 0.4 μl *Taq* DNA polymerase (5 U/μl). First-round PCR was performed in the following conditions: hot start for 5 min at 96 °C, followed by 35 cycles of PCR consisting of 1 min at 95 °C, 2 min at 55 °C, and 2 min at 72 °C. For the second round of PCR, 10 μl of the first-round samples was used and the conditions for the PCR was the same. PCR products (423 bp) were identified by agarose electrophoresis and purified with 3S PCR Purification Kit (Shengneng Biocolor) to eliminate impurities such as dNTPs, primers, polymerase, etc. The DNA was cloned into pGEM-T vector using T_4DNA ligase (Promega, Madison, WI), and plasmid DNA was isolated with the QIAprep Spin Miniprep kit. Clones containing the appropriate inserts were sequenced using BigDye Terminator Cycle Sequencing Ready Reaction(PE) and analyzed by automated DNA sequencing on an ABI 377 capillary sequencer. For the experimental group, six clones corresponding to each sperm sample of four male parents' mice were selected, therefore total 24 clones were picked up and sequenced. For the control group, two clones corresponding to each male parent mouse were selected, therefore total eight clones were picked up and sequenced. For the fetal liver samples of the first offspring, four clones corresponding to each sample of eight fetal mice were selected, therefore total 32 clones were picked up and sequenced. The number of fetal mice and clones to be sequenced in the control group was the same as the experimental group.

Results

The analysis of methylation patterns for CpG islands of H19 DMR in the sperms of male parent mice

We examined a total of 16 CpG sites in the 5′ end of H_{19} (GenBank Accession No. AF049091). Totally eight clones from control group were sequenced, and the nucleotide sequence of one clone from No. 1623 to No. 1670 is shown in Fig. 1A (No. 445–No. 582 in sequencing

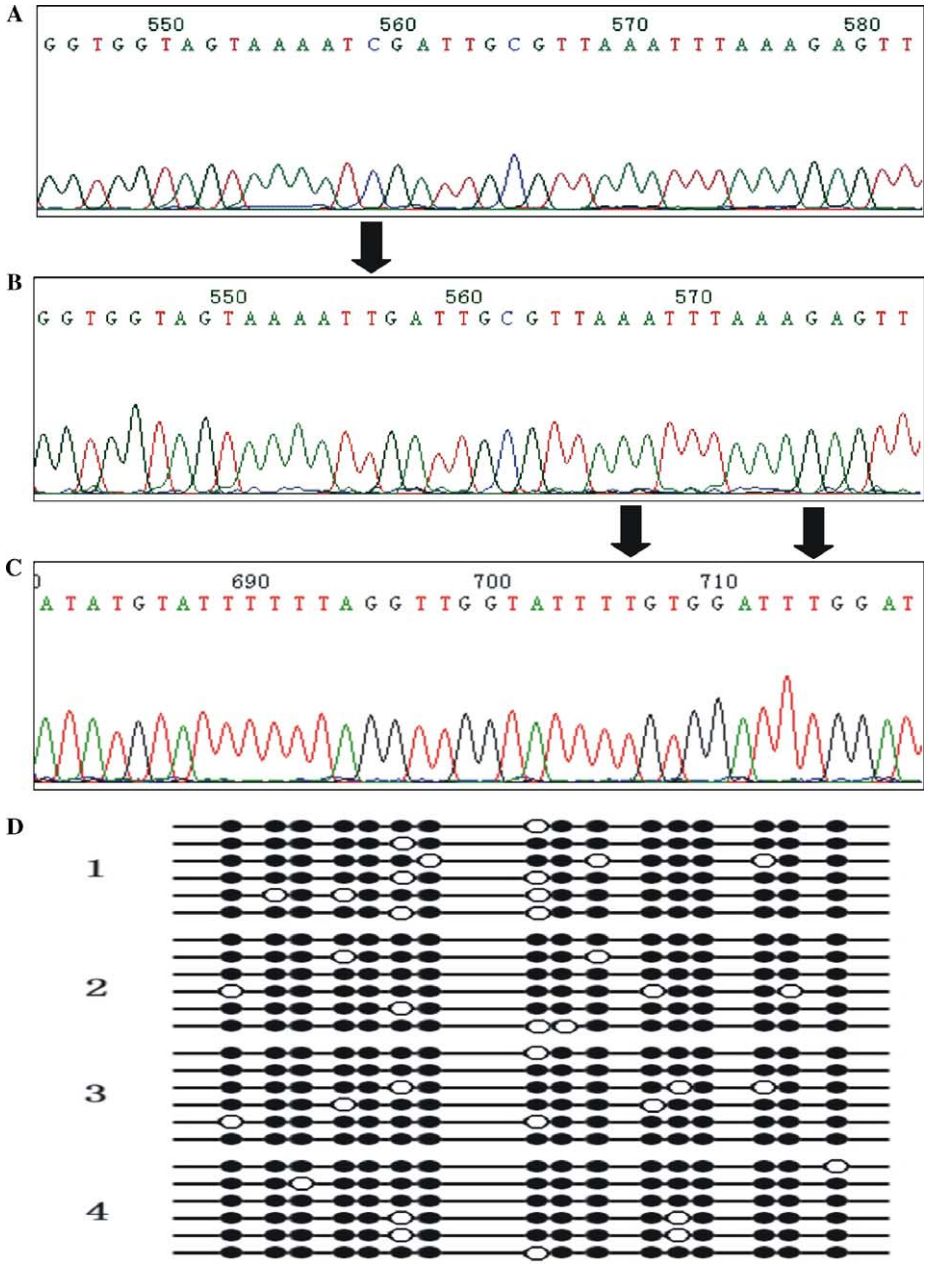

Fig. 1. The effects of X-ray to the methylation patterns in CpG islands of H19 DMR in mice sperms. The sperm DNA samples were treated with sodium sulfite, and PCR products were subsequently cloned into T vector and sequenced. (A) Nucleotide sequence of one clone from No. 1623 to No. 1670 in control group. (B,C) Sequencing results of two clones, respectively, in experimental group. (D) The patterns of CpG islands in 24 experimental clones.

electropherogram). The cytosines in CpG islands remained unchangeable, whereas the cytosines in non-CpG islands were converted to thymidines. Totally 24 clones were obtained and sequenced from the experimental group. Of them, 19 clones presented the conversions from cytosines to thymidines in CpG islands. Fig. 1B shows the partial electropherogram of the nucleotide sequence of No. 2 clone from No. 1623 to No. 1670 (No. 442–No. 579 in sequencing electropherogram) in one mouse. The conversion from cytosine to thymidine appeared in the sixth CpG island (No. 1637 nucleotide). Fig. 1C shows the partial electropherogram of the nucleotide sequence of No. 6 clone from No. 1759 to No. 1796 (No. 681–No. 718 in sequencing electropherogram) in No. 2 mouse of experimental group. The cytosine in the eighth and ninth CpG islands has been converted from cytosine to thymidine (No. 1784 and No. 1792 nucleotides, respectively). The patterns of CpG islands in all 24 clones of experimental group were summarized in Fig. 1D. Dot indicates the methylated cytosine; circle indicates the conversion from unmethylated cytosine to thymidine. Fig. 1D indicates the conversions of cytosine to thymidine in CpG islands found in 19 clones, although the number and site were uncertain. There were four clones with three CpG alternations, eight clones with two CpG alternations, seven clones with one CpG alternation, and five clones without CpG alternation.

The analysis of methylation patterns for CpG islands of H_{19} DMR in the fetal livers of the first-generation offspring mice

To clarify whether the alternations of CpG methylation patterns induced by radiation could be maintained in the first offspring, we detected the CpG methylation patterns of H_{19} DMR in fetal liver tissues of F_1 offspring mice and compared them with those of male parent sperms, and the relations to the male parent sperms. Since the genome of mouse fetal liver is diploid, derived from both male parents, the sequencing results of sodium sulfite-treated clones would be the complex of the parents' alleles. We must first of all distinguish the sequencing results of double parents' derived alleles. The female derived clones were decided based on the conversions from cytosine to thymidine in all sequencing results, because no cytosine methylation occurred in the H_{19} CpG islands of female parents. However, in the H_{19} DMR of male parents, all cytosines in the CpG islands were methylated, the cytosines in sequencing results remaining unchangeable. Even though some cytosines in CpG islands could not get converted to thymidines in spite of a radiation, the sequencing results in the sites of CpG islands should be a hybrid of cytosines and thymidines, therefore, we regarded them as male parents' alleles. With this project, Wu et al. [18] had studied the influence of chemical materials on the methylation of imprinted genes during the development of fetal mice.

Fig. 2A shows the partial DNA sequencing results (No. 1783–No. 1821 nucleotides) of H_{19} gene in the chromosome of female parent in the fetal livers of the first-generation offspring mice and indicates that all cytosines were converted to thymidines in the eighth, ninth, and tenth CpG islands. We obtained 17 and 15 female parent clones from 32 clones of experimental group and 32 clones of control group, respectively.

Fig. 2B shows the partial DNA sequencing results (No. 1783–No. 1821 nucleotides) of H_{19} gene in male chromosome of control group and indicates that cytosines in CpG islands remained unchangeable, but the cytosines in non-CpG islands were converted to thymidines. Total 15 male parent clones in experimental group were obtained. One nomination pattern was defined because of excessive clones and their complicated derivation. Pa-b-c: P represents the clone of a male parent chromosome, a represents the serial number of male parent mice, b represents the serial number of fetal mice, and c represents the serial number of clones. For example, Fig. 2C shows the sequencing result of the P2-2-3 clone in the experimental group, which was derived from No. 2 male parent mouse, which demonstrated the conversion of cytosines to thymidines in CpG islands. Although some cytosines in CpG islands were converted to thymidines (thick arrowhead indicates the 10th CpG island), some cytosines still remained unchangeable (thin arrowhead indicates the ninth CpG island). The sequencing results of 15 clones of fetal mice in experimental group are summarized in Fig. 2D, the array sequence from top to bottom indicating the serial number of male parent mice →the serial number of fetal mice →the serial number of clones. Some cytosines in CpG islands were converted to thymidines in different clones, and the number and sites were not completely the same. There were three clones without CpG alternation, two clones with three CpG alternations, six clones with two CpG alternations, and four clones with one CpG alternation.

After comparing the methylation patterns of CpG islands in male parent sperms (shown in Fig. 1D) with that of male parent-derived chromosome in fetal mice (shown in Fig. 2D), we found two CpG island patterns which did not exist in male parent sperms (Clone P 1-1-3 and Clone P 1-1-4), the CpG island patterns in other 13 clones which existed in male parent sperms.

Discussion

We chose the BALB/c inbred lines as an experimental model because they share 99.9% similarity of genetic background, which could reduce the effects of genetic differences on experimental results. After the DNA samples were treated with sodium sulfite, the methylated cytosine remains as cytosine, whereas the non-methylated cytosine will be converted to thymidine, thus we can deduce whether the methylation patterns have been disturbed in CpG islands [19]. Since the isolated DNA was an assembly of many sperms, the direct sequencing of PCR products could not indicate the methylation pattern of a single sperm, so the PCR products were cloned into pGEM-T vectors, and monoclonal DNA was sequenced. The sequencing results of 24 clones in experimental group are shown in Fig. 1D. There were unmethylated cytosines in CpG islands of 79.2% (19/24) clones, including four clones with three CpG island alternations, eight clones with two CpG island alternations, and seven clones with one CpG island alternation, indicating that the ionizing radiation could interfere with the imprinting process of a majority of germ cells. The distributions of methylation changes were confused in 16 CpG islands without any rules, indicating that the interference of ionizing radiation with the imprinting process was a random event. The number of methylation changes was not beyond 3, 78.9% of clones undergoing 1–2 methylation changes. The phenomenon could be explained below. First, although the imprinting was a gradual process, it had started before the mouse was born, and quite a few parts of imprinting had been finished in the period of meiosis; second, the dosage of radiation was too low to change the methylation patterns of a majority of CpG islands; third, part of alternations in CpG islands caused by radiation might be resumed because the whole-body irradiation was applied as 0.1 Gy per day with the dosage rate of 2 Gy/min for 3 s. After 13 days of radiation treatment, the sperm samples were taken after 20 days for further test, therefore, there was enough time to resume.

Moreno et al. [20] reported the methylation changes caused by radiation, in which the hypomethylated pattern of germ cell DNA in male fetal mice was converted to a

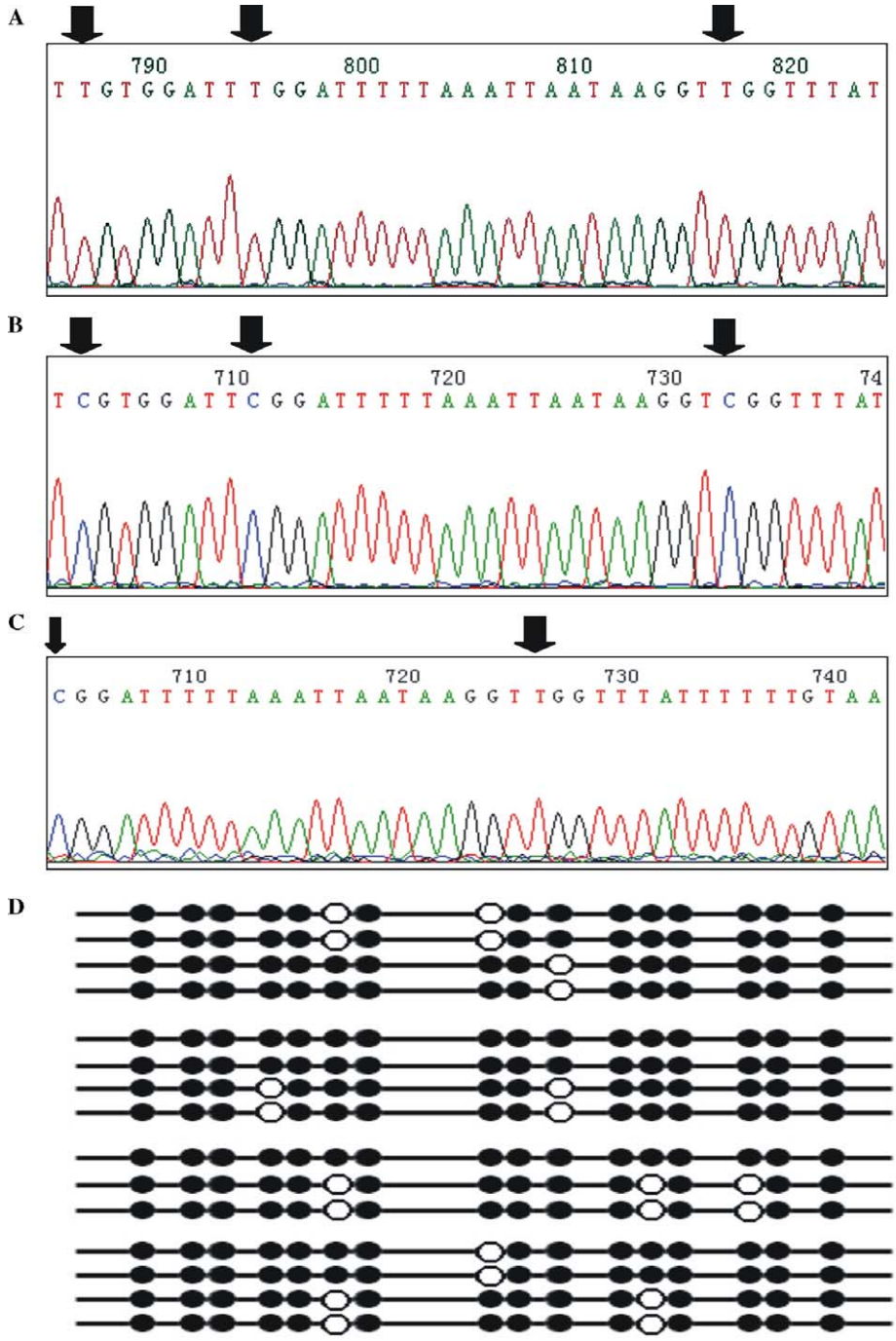

Fig. 2. The fetal liver DNA of first-generation offspring mice was treated with sodium sulfite, and PCR products were subsequently cloned into T vector, and sequenced, indicating the changes of methylation patterns in H19 MDR. (A) The partial DNA sequencing results of H19 gene in the chromosome of female parent, (B) The partial DNA sequencing results of H19 gene in male chromosome of control group, (C) The sequencing result of male parent in experimental group, (D) summarized the patterns of CpG islands by sequencing the paternal DNA of 15 clones in the experimental group.

hypermethylated pattern detected with anti-methylated cytosine antibodies, when the maternal mice were subjected to 1.5 Gy of radiation treatment, 19 days after copulation. Their research data showed that 1.5 Gy of acute radiation could improve the total genomic methylation levels of germ cells. Kovalchuk et al. [11] treated male mice with X-ray, applied as 5 cGy per day for 10 days, and 50 cGy of acute radiation once, respectively, and found that chronic radiation could remarkably reduce the total genomic methylation levels of male mice's muscle. The methylation patterns of p16 gene in mice's muscles and livers were also analyzed using the methylation-specific PCR technique, and methylations were found in the promoter region of liver p16 gene, but similar changes were not discovered in muscle tissue, indicating that the effects of ionizing radiation on methylation were related to sex, tissue, and dosage. The popularity of methylation unbalance in carcinogenesis indicates the broad genomic hypomethylation and part

hypermethylation of CpG islands [21]. Dote [22] reported that zebularine, the methylation inhibitor, could improve the tumor cell's sensitivity of radiation treatment; however, the relationship between the carcinogenesis and the changes of genomic methylation level caused by ionizing radiation would still need to be further studied. Kovalchuk's work [10,12] indicated that methylation changes caused by ionizing radiation were related to the activities of methylase DNMT3a and DNMT3b. The methylation reaction was accomplished with the cooperation of DNMT3a and DNMT3b [23]. The gene variations of DNMT3a and DNMT3b could affect the normal development, as Okano et al. [24] indicated that the mice lacking DNMT3a showed dystrophy and died mostly in 4 weeks; mice lacking DNMT3b were mostly aborted in the embryo stage. Our study revealed that X-ray could induce part of cytosines in CpG islands to become unmethylated during the imprinting formation. Whether the radiation inhibits the activities of DNA methyltransferase is still to be explored.

The methylations happened only in the ICR of mice sperms, not in that of mice ova [25,26]. The methylation pattern in sperms remained unchangeable after fertilization in spite of the influence of subsequent demethylation, whereas the CpG islands in ova maintained unmethylation [27]. After embryo formation, the paternal alleles were closed due to methylation, only the maternal alleles were expressed, and diploid fetal cells kept in the stage of unilateral alleles' expression. According to this principle, the methylation changes of sperm H_{19} gene in ICR caused by ionizing radiation should remain unchangeable in fetal development, the paternal allele of H_{19} in diploid fetal mice cells should exhibit a methylation pattern that was derived from male parent, in which methylated and unmethylated cytosines existed simultaneously in CpG islands, whereas only one unmethylated pattern existed in maternal alleles. There were no direct evidences to confirm the deduction hereinbefore, so we arranged for radiation-treated male mice to copulate with normal female mice. We compared the methylation patterns of H_{19} ICR in parent sperms with those in fetal mice liver to clarify whether the H_{19} methylation alternations in paternal sperms induced by radiation could be inherited to offspring and remain unchangeable.

The experimental results indicated that in control group, the CpG islands in H_{19} of maternal chromosome were in the state of non-methylation (Fig. 2A), whereas in paternal chromosomes, cytosines in CpG islands of H_{19} ICR in mature sperms were totally methylated and the imprinting was formed (Fig. 2B). These data given hereinbefore were in accord with previous researches [16]. Thirty-two clones in the experimental group were divided into three circumstances: (1) There were 17 clones with only thymidines in CpG islands, thus we deduced that these clones were derived from maternal alleles. (2) Three clones were derived from normal sperms that were not interfered by radiation in the imprinting process, in which cytosines in CpG islands remained unchangeable and cytosines in non-CpG islands were converted to thymidines. (3) All cytosines in non-CpG islands were converted to thymidines. Some of the cytosines in CpG islands remained unchangeable, while some of them were converted to thymidines. These kinds of clones were derived from the sperms that were interfered by radiation in the imprinting process (Fig. 2C). The latter 2 circumstances were the sequencing results of paternal chromosome in fetal mice livers and summarized in Fig. 2D. Two clones (PCL1-1-1 and PCL1-1-2), in which the 6th and 8th CpG islands were interfered, respectively, and two clones (PCL1-2-1 and PCL1-2-2), in which the 10th CpG island was interfered, were obtained according to the sequencing results of the paternal chromosomes of two fetal mice, which were born after No. 1 male mouse was copulated. Compared with the sequencing results of the sperms of No. 1 male mouse shown in Fig. 1D, two patterns that 6th and 8th CpG islands were interfered other than the pattern that 10th CpG island was interfered existed in sperms. Clones derived from the same fetal mouse show the same sequencing results. There were the same methylation patterns between the paternal chromosomes of fetal mice and the sperms of their No. 2, 3, 4 male parent mice, respectively. The limited number of randomly selected clones might cause more clones of maternal chromosomes than those of paternal chromosomes, so it was noticeable that only one paternal chromosome sequencing result was obtained from one fetal mouse, which was born after No. 3 male mouse was copulated. Comparing the methylation patterns of H_{19} CpG islands of paternal chromosomes in fetal livers with those of male parent sperms, we deduced that the methylation patterns of fetal mice were derived from those of the male parent sperms, since there were almost the same methylation patterns except for one clone of fetal mouse. For one fetal mouse, born after No. 1 male mouse was copulated, the methylation pattern of H_{19} CpG islands in paternal chromosomes of liver tissue had not existed in male parent sperms, probably because the number of clones was too low, and some methylation patterns of H_{19} CpG islands in sperms have not been found yet.

H_{19} encoded an RNA, the function of which was not clear. Hao et al. [28] thought that H_{19} could suppress the tumor, and its overexpression would resume rhabdomyoblastoma G401 of the embryo; however, Kim et al. [29] observed 15 cases of ovarian cancers and found that double alleles were expressed in 62% of ovarian cancers, which did not support the theory that H_{19} could suppress carcinogenesis. Ariel et al. [30,31] had studied hepatocellular carcinoma and found that RNA encoded by H_{19} was actually a tumor fetal RNA, which promoted lot of genes in becoming up-regulated. Because these up-regulated genes were related to invasion, metastasis, and angiology, the up-regulation of H_{19} was closely related to carcinogenesis and development. Lottin et al. [32] found that H_{19} RNA was overexpressed in myometrium and stroma during the formation of endometrioma, and regarded it as cytopathological hyperplasia, indicating that H_{19} RNA could promote cytocarcinogensis. We found in this work that some cytosines in CpG islands of H_{19} gene were not methylated due to ionizing radiation. The H_{19} gene should be

up-regulated when it was transferred to the first-generation offspring, and induced expression changes of many downstream genes because the expression product was RNA (not a protein), which caused more complicated biological effects. The genetic effects of radiation interference to imprinting formation from parent to offspring were not deeply observed, the imprinted genes with clear biological effects should be chosen in further studies to illustrate the effects of radiation.

Although the data of imprinting formations interfered by ionizing radiation were not enough, it was clear as Schofield [33] stated, the nature of the imprinted genes was the unilateral expression of allele, therefore the imprinted gene mutation caused by ionizing radiation would easily induce genomic unbalance, leading to genetic diseases and tumors.

Acknowledgments

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