[Frontiers in Bioscience E2, 165-170, January 1, 2010]

Acrylamide disturbs genomic imprinting during spermatogenesis

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1. ABSTRACT

Acrylamide (ACR), a carcinogen for rodents and humans, exists widely in the human living environment and heat-treated carbohydrate foodstuffs. Increasing evidence has demonstrated that ACR can cause chromosomal damage in somatic cells and mutagenesis. However, little is known about whether ACR can disturb genomic imprinting during spermatogenesis. We investigated the effects of ACR on methylation patterns of rat sperm genes. The results showed that after oral administration of ACR to rats for two weeks, methylation of some cytosines in the CpG islands of the differentially methylated region (DMR2) of sperm gene insulin-like growth factor II (Igf2), which is still present at the 19th day, disappeared on the 35th day. Furthermore, the extent by which ACR causes methylation defects varies in animals. Our findings indicate that mitotic spermatogonia and primary spermatocytes are sensitive to ACR-induced genomic imprinting aberration, suggesting that ACR predominantly interferes with the remodeling process in spermatogenesis.

2. INTRODUCTION

ACR is a reactive and highly water-soluble vinyl monomer widely present in our living environment. Recently, significant levels of ACR have been found in foodstuffs heated with high temperatures (1-3). Thus, humans can be exposed to ACR through their diets, raising wide concerns about its safety.

ACR has been proven to be carcinogenic in rodents and a probable carcinogen in humans causing genotoxicity including micronuclei, chromosomal aberration, sister chromatid exchanges and mitotic disturbances (4, 5). Notably, ACR and its metabolites have a strong affinity for mammalian sperm cells. It can induce genetic damage with great efficiency at the late stages of spermatid maturation in the epididymis. The occurrence of mutation in germ cell can be transmitted to subsequent generations causing genetic diseases (6).ACR has been shown to affect various reproductive parameters in mice including, a decrease in the amount of sperms or an increase in the number of morphologically abnormal

sperms (7). Furthermore, ACR can cause abortion mediated by micronuclei of spermatocytes and spermatids. Interestingly, ACR-induced genetic aberrations mainly occur during the post-meiotic stages of spermatogenesis, such as speremato zona, late spermatids and spermatogonia in mice (7).

During gametogenesis, imprinted genes are epigenetically marked so that offspring will exclusively express either paternal or maternal allele. DNA methylation is one of the most important epigenetic markers. The methylation of CpG islands is frequently found in the promoter regions of genes and the imprinting control region (ICR). The variations of methylation pattern at CpG islands, the differentially methylated region (DMR), usually determine differential gene expression between paternal and maternal alleles (8). The establishment of paternal-specific methylation (methylation pattern in the ICR) is a continuous process occurring in mitotically dividing spermatogonial stem cells, as well as in the derived mitotically dividing spermatocytes (9). Imprinting defects caused by exogenous factors during spermatogenesis may result in gene expression problems, which can be inherited by offspring (10). ACR can induce mutagenesis of germ line cells. However, the effect of ACR on genetic imprinting during spermatogenesis is poorly understood.

Imprinted gene of insulin-like growth factor II (Igf2) is subjected to epigenetic modification and parental imprinting, leading to a predominant expression of paternal allele in humans, mouse and rat (11-13). The DMR2 lies in the 3' part of the coding region and is differentially methylated in germ cells. Its methylation is usually lost during the early pre-implantation of the embryo and is reestablished at the late stage of spermatogenesis (14, 15), providing an ideal model for investigating gene imprinting during spermatogenesis.

In the present study, we attempted to explore whether ACR exposure could interfere with the genomic imprinting during spermatogenesis. Sprague-Dawley (SD) rats were treated orally with different daily doses of ACR for a period of two weeks. The methylation pattern of Igf2 DMR2 was examined on the 19^{th} and the 35^{th} day post-treatment, which reflects the various spermatogenesis stages. Our results indicate that ACR induced stage-specific imprinting defects in sperms.

3. MATERIALS AND METHODS

3.1. Chemicals

ACR was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in physiological saline solution.

3.2. Animals

Adult male (body weight, 350-400 g) SD rats were purchased from the Beijing laboratory animal center (Beijing, China). Rats received food and water *ad libitum* and were maintained on a 12-hour light/dark cycle in a temperature-controlled room. After acclimation for one week, male rats were randomly divided into three groups and orally treated with ACR at doses of 5 mg/kg (n=6), 50 mg/kg (n=6) or saline (control, n=3) daily for two weeks. These rats were sacrificed on day 19 or 35 after treatment according to the cycle (12.9 days per cycle) of seminiferous epithelium.

3.3. Bisulfate-sequencing PCR (BSP)

4 μg of sperm DNA in 50 μL Tris-EDTA (TE) was denatured with 5.5 μL 2 mol/L NaOH for 15 min at 37 ºC. Then the sperm DNA was treated with 30 μL of 10 mmol/L hydroquinone (Sigma, USA), 520 μL of 3 mol/L sodium bisulfate (Sigma, USA) at pH 5.0, and 200 μL of mineral oil as a vapor barrier. The mixture was incubated for 16 h at 52 ºC for bisulfate modification. The modified genomic DNA was purified using ~~the~~ Wizard DNA purification resin according to the manufacturer's instructions (Promega, Madison, NJ). The purified DNA was eluted in 50 μL of water and treated with NaOH for 10 min at room temperature, followed by DNA precipitation with ethanol. The DNA was dissolved in milli-Q water and stored at -70 ºC. The unmethylated cytosine residues in the genomic DNA were deaminated to uracil, leaving 5-methylcytosine intact.

Igf2 DMR2 was amplified by PCR using Master PCR mix (Shenergy Biocolor Company, Shanghai, China). Two steps of touchdown PCR were performed with nested primer pairs. The primers for PCR were as follows: first step, 5'-AAC TAA AAT TAT CTA TCC TAT AAA AC-3' (sense) and 3'-ATTG ATG GAT TTA TAT TGT AGA ATT AT-5' (antisense); second step, 5'-GGA ATT CCC TAT AAA ACT TCC CAA ACA AC CTT CAA A-3' (sense) and 3'-GGA ATT CCT GAT TTA TTG ATG GTT GTT GGA TAT TT-5' (antisense). The cycling conditions were denaturation at 96 ºC for 5 min, then 10 touchdown cycles of 94 ºC for 30 s, 58 ºC -50 ºC for 30 s and 72 ºC for 30 s, 20 cycles of 94 ºC for 30 s, 50 ºC for 30 s and 72 ºC for 30 s and a final extension of 72 ºC for 7 min. The PCR products were separated by 1.2 % agarose gel and purified using the gel purification kit (Promega, USA). The purified PCR products were subcloned into the pGEM-T vector and directly sequenced (ABI3730 sequencer) for the analysis of methylation patterns. Sequences with more than 95% bisulfate conversion efficiency were used for analysis.

4. RESULTS

4.1. PCR amplification of Igf2 DMR2

Firstly, genomic DNA was extracted from rat sperm using routine methods and the CpG island region was amplified with specific primers. A 731-bp PCR product was obtained (Figure 1) and subjected to sequencing. The sequence of amplified fragment was identical to that provided by DNAMAN (Version 5.2.2, Lynnon Biosoft, Quebec, Canada) from the NCBI database. The precise sequence of the amplified CpG island region provided a unique basis for subsequent analysis.

4.2. Effect of ACR on the methylation patterns of sperm CpG islands in Igf2 DMR2 19 days post-treatment

Bisulfate treatment can convert unmethylated cytosines to uracils (identified as thymine in sequencing)

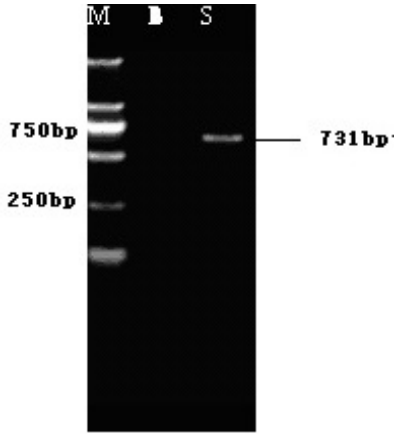

Figure 1. Bisulfate PCR analysis of Igf2 MDR2 fragments. Sperm genomic DNA was extracted, treated with bisulfate and subjected to PCR amplification. The PCR products were separated by agarose gel and visualized using ethidium bromide. M, DNA molecular weight marker; B, control PCR products in the absence of DNA templates; S, experimental PCR products of Igf2 DMR2.

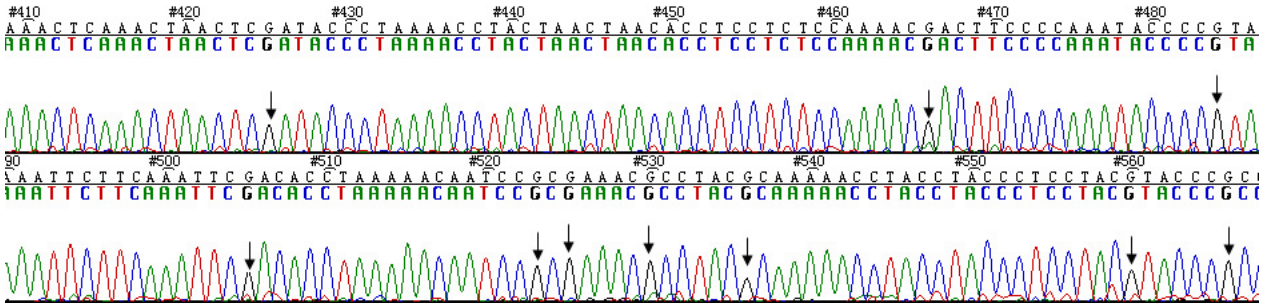

Figure 2. Sequence analysis of bisulfate PCR products. Sperm cells were collected on the 19th day post acrylamide treatment and genomic DNA was applied to bisulfate PCR amplification. The purified PCR products were subjected to sequencing using an ABI3730 sequencer. Data were representative of xxx clones. The differentially methylated CpG sites were indicated with black arrows.

but does not affect the methylated ones allowing examination of the effect of ACR treatment on the methylation patterns of CpG islands in Igf2 DMR2 of rat sperm. Rats were sacrificed on the 19th day after ACR treatment (late stage of meiosis). Analysis of amplified CpG island sequences of Igf2 DMR2 from experimental and control rats revealed that there was no detectable uracil in the CpG islands, although many cytosines outside CpG islands had been converted to uracils; this indicates that all cytosines in the CpG islands were methylated (black arrow in Figure 2). Moreover, sequences of all the clones from rats that had been treated with different doses of ACR were identical to those of the control group, suggesting that ACR treatment did not affect the methylation patterns of sperm CpG islands in Igf2 DMR2 at the late stage of meiosis regardless of ACR dose.

4.3. Effect of ACR of ACR on the methylation patterns of sperm CpG islands in Igf2 DMR2 35 days post-treatment

To determine whether treatment with ACR could affect the methylation patterns of sperm CpG islands in Igf2 DMR2 at early stage of spermatogenesis, sperms from rats were isolated and the methylation patterns on the 35th day after ACR treatment were analyzed. Nine clones from three rats (three clones per rat) were analyzed. While control clones showed no single uracil in the CpG islands, those from ACR-treated rats displayed many uracils in the region, indicating that ACR treatment did not methylate these cytosines (Figure 3A, B). The extent to which ACR induced methylation defects in different clones was variable. Some clones displayed approximate normal pattern of CpG island methylation, similar to that from the control group while others lost as high as 40% of cytosine methylation at different sites. Surprisingly, treatment with a lower dose (5 mg/kg) of ACR induced a higher proportion of unmethylated cytosines as compared to those with the higher dose (50 mg/kg) ($p<0.05$). The average percentage of unmethylated cytosine in rats treated with ACR at 5 mg/kg dose was 18.6%, while the one at 50 mg/kg dose was 7.08% (Figure 3C). The detailed patterns of CpG islands in 18 experimental clones from these two groups of rats treated with ACR are shown in Figure 3D. Collectively, treatment with ACR changed epigenetic imprinting by modifying the cytosine methylation of CpG islands in Igf2 DMR2 of rat sperm.

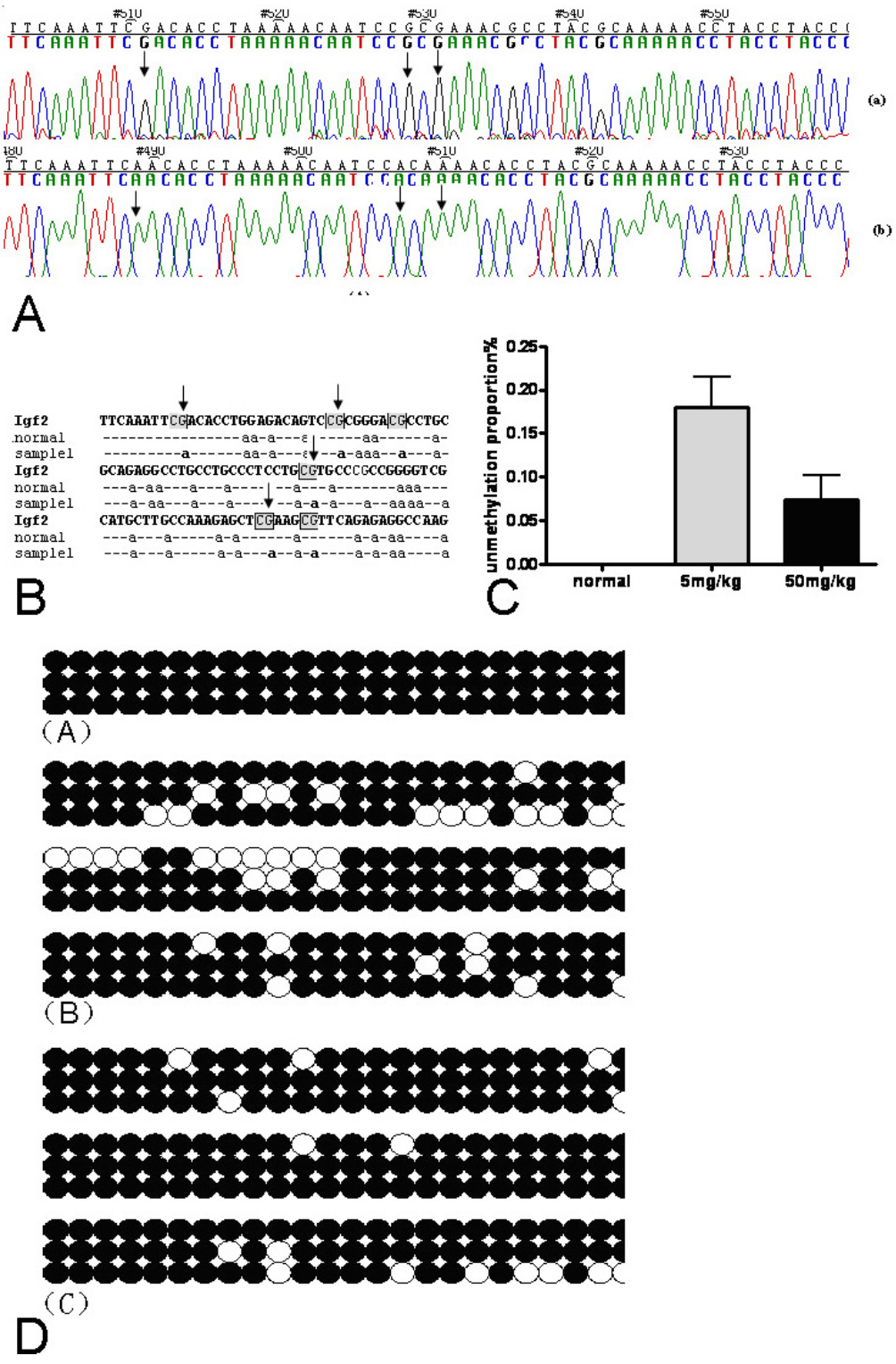

Figure 3. Analysis of the methylation patterns of sperm CpG islands in the Igf2 DMR2. Groups of rats were treated with acrylamide and their sperm was collected 35 days after acrylamide treatment. The PCR products of sperm CpG islands in the Igf2 DMR2 were sequenced. (A) DNA sequencing analysis of Igf2 DMR2 fragments. a, control group; b, acrylamide treatment group; (B) sequence alignment of (A); (C) proportion of unmethylated CpG sites per 25 total CpG islands in Igf2 DMR2; (D) the detailed methylation patterns of all 18 clones. White circles indicate unmethylated sites and black ones refer to the methylated sites. a, control group; b, acrylamide at 5 mg/kg; c, acrylamide at 50 mg/kg.

5. DISCUSSION

Gene expression is not determined solely by DNA base sequence but also by epigenetic phenomena (16). Epigenetic disorders caused by chemical or physical agents have the potential to produce adverse effects by causing heritable changes to the genome that result in heritable alterations in the phenotype (17). Genomic imprinting is an epigenetic phenomenon that, in most cases, is believed to occur during gametogenesis. DNA methylation plays a predominant role in gene imprinting, which appears to be involved in transcriptional regulation controlling aspects of development, tissue-specific gene expression, expression of imprinted genes and silencing of transposable elements (18). In particular, genes are differentially marked by methylation in the developing male and female germ cells to ensure parent-of-origin-specific expression in the offspring. Altered DNA methylation has been shown to contribute to carcinogenesis and other neuronal and developmental disorders (19). Because ACR is widely present in our environment and has genetic interference with sperm development, it is of great importance to determine the effect of ACR on DNA methylation.

The Duration of the cycle of seminiferous epithelium in SD rats is 12.9 days and it generally takes 4 cells for a spermatogonia to develop into spermatids. Therefore, SD rats are considered as an ideal model for evaluating the genetic toxicity of ACR. In the present study, while there was no detectable change in the methylation pattern of CpG islands in Igf2 DMR2 of rat sperm collected at the 19th day after ACR treatment, a loss of gene imprinting was observed on the 35th day. Apparently, ACR treatment predominately affected spermatogonia and early spermatocytes when paternal-specific methylation was established. ACR may cause a loss of imprinting in Igf DMR2 during the remodeling stage of spermatogenesis. In somatic cells, three distinct DNA cytosine methyltransferases (Dnmts) Dnmt1, Dnmt3a, and Dnmt3b control DNA methylation patterns (20). However, Dnmt3a and Dnmt3L have been shown to play crucial roles during *de novo* acquisition of DNA methylation patterns in male germ cells (21). Kaneda and colleagues also reported that male mice with conditional mutant of Dnmt3a^{-}, but not Dnmt3b^{-}, showed severely impaired spermatogenesis and lack of methylation at two of three paternally impaired loci in spermatogonia (22). However, during spermatogenesis, Dnmt3L could be involved in the *de novo* methylation of genomic sequences other than ICRs and impaired genes. Indeed, a recent study showed that inactivation of Dnmt3L induced meiotic failure in spermatocytes and spermatogenesis arrest (20). Because ACR has been demonstrated to affect spermatocyte maturation and spermatogenesis, it is possible that ACR may also affect the activity of Dnmt3a or Dnmt3L to disturb sperm imprinting. The precise mechanisms underlying genetic toxicity of ACR remains to be further examined.

It is not surprising for us to detect variation of methylation changes in different clones generated from rats treated with ACR. Resistance to ACR may reflect that some sperm from late stage of maturation were contaminated in the collected samples, due to technical difficulty in isolating homogeneous sperm samples. In fact, sperm cells at different stages of spermatogenesis coexist in the epididymis. Surprisingly, however, lower dose of ACR induced a greater loss of imprinting in Igf2 DMR2 compared to higher dose. Since the methylation is closely associated with higher incidence of mutation, exposure to lower dose of ACR may be more powerful in inducing genomic instability. Finally, because high temperature-treated carbohydrate foodstuffs contain low levels of ACR, high consumers of these foodstuffs may be at a high risk to develop cancers and other genetic disorders.

In summary, our study revealed that ACR can induce perturbation in methylation imprinting. Since methylation patterns are usually inherited from the parents to the offspring, it is important to evaluate the methylation variations in terms of safety from hazardous effects of ACR. Although little is known about the mechanisms underlying the genetic toxicity of ACR, our results provide preliminary evidence to demonstrate that ACR caused methylation aberrations, and how an assessment of methylation status can provide important information as a component of an overall safety assessment.

6. ACKNOWLEDGEMENTS

Zhuoqun Wang, Shuang Lu contributed to this manuscript This work was supported in part by the major state basic research development program of China (2001CB5103), the National infrastructure program of Chinese Genetic Resources (2005DKA2130). We are grateful to Professor Zhong Shudong for his advice regarding to the design of the experiment. We also thank Liwen Editing Company for editing the English Contents.

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Abbreviations:ICR: imprinting control region; DMR: differentially methylated region; Igf2: insulin-like growth factor II; SD: Sprague-Dawley ; BSP: Bisulfate-sequencing PCR; TE: Tris-EDTA; Dnmts: DNA cytosine methyltransferases

Key Words: ACR; Genomic Imprinting; Spermatogenesis

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