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Genomic Alteration of Bisphenol A Treatment in the Testis of Mice

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Abstract

Bisphenol A (BPA) is commonly used in the production of pharmaceutical, industrial, and housing epoxy, as well as polycarbonate plastics. Owing to its extensive use, BPA can contaminate the environment either directly or through derivatives of these products. BPA has been classified as an endocrine disruptor chemicals (EDCs), and the primary toxicity of these EDCs in males involves the induction of reproductive system abnormality. First, in order to evaluate the direct effects on the Y chromosome associated with reproduction, we evaluated Y chromosome abnormalities using a Y chromosome microdeletion detection kit. However, we detected no Yq abnormality as the result of BPA exposure. Secondly, we performed high-density oligonucleotide array-based comparative genome hybridization (CGH) to assess genomic alteration as a component of our toxicity assessment. The results of our data analysis revealed some changes in copy number. Seven observed features were gains or losses in chromosomal DNA (*P*-value<1.0e-5, average log2 ratio>0.2). Interestingly, 21 probes of chr7:7312289-10272836 (qA1-qA2 in cytoband) were a commonly observed amplification (*P*-value 3.69e-10). Another region, chr14:4551029-10397399, was also commonly amplified (*P*-value 2.93e-12, average of log2 ratios in segment>0.3786). These regions include many genes associated with pheromone response, transcription, and signal transduction using ArrayToKegg software. These results help us to understand the molecular mechanisms underlying the reproductive effects induced by BPA.

Keywords: Bisphenol A, Array-CGH, Genomic alteration, Toxicity, Pheromone, Microdeletion

Bisphenol A (BPA) is primarily utilized to make epoxy and polycarbonate plastics, and those products have been utilized in recent decades. It can contaminate the environment either directly or via the degradation of BPA-containing products. Notwithstanding the broad utility of BPA, the chemical has been classified as toxic to human health and the environment, and has been implicated in several negative health effects, including endocrine disruption[1,2]. In previous studies, it has been noted that endocrine disruption chemicals (EDCs) may function act as disruptors of normal endocrine function. EDCs perform important roles in male and female reproductive functioning, as well as in development, differentiation and growth. In recent studies, EDCs have been demonstrated to influence reproduction in all studied animal groups, to induce genetic aberrations. Although a great deal of research has been conducted in this area, the molecular mechanisms underlying these reproductive effects remain poorly understood[3-5].

From the genomic perspective, the deletion of the long arm of the Y chromosome (Yq) is frequently associated with male infertility. Recent genetic studies of male infertility have demonstrated that the long arm of the Yq harbors at least 15 families, some of which have been shown to be necessary for spermatogenesis[6,7]. We hypothesize that male reproductive abnormality may arise as a problem of Y chromosome-microdeletion as the consequence of exposure to BPA. We assessed genomic abnormalities of the Y chromosome using a multiplex-PCR-based Y chromosome microdeletion detection kit in *in vitro* systems. However, we ultimately detected no Y chromosome abnormalities.

Genetic approaches, including comparative genomic hybridization (CGH), perform an important function

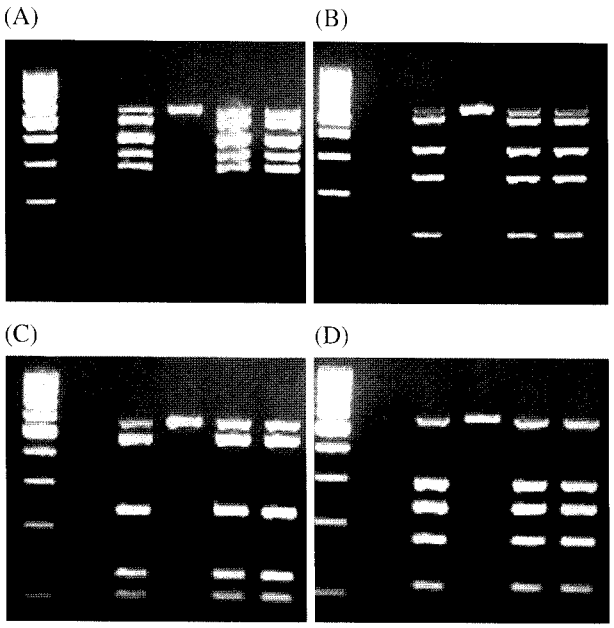

Figure 1. Result of Y chromosome multiplex PCR. All 32 probes were detected with a Y chromosome microdeletion detection kit. (A) STS1 consist of ZFX, sY14, sY254, sY86, sY127 marker, (B) STS2 consists of ZFX, sY14, sY84, sY134, sY255 marker, (C) STS3 consist of ZFX, SPGY2, sY158, sY152, sY147 marker, (D) STS4 consists of ZFX, sY157, sY242, sY130, sY124 marker. All 1st lanes are a 100 bp ladder marker, the 2nd lanes are no mixture and DNA, the 3rd lanes are positive controls (male DNA), the 4th lanes are negative controls (female DNA), and the 5-6th lanes are BPA-treated sample DNA.

in biological studies. Chromosomal variations or copy number variations are associated with aberrant gene and protein expressions[8]. Secondly, toxicity assessments, such as the array-based CGH method utilized in this study, can be used to measure the copy number values of the genes. In an array-based CGH, DNA are hybridized onto the slide, and the copy numbers of the genomes can be measured. Recently, the resolution of array-based CGH has been significantly improved. Comprehensive probe coverage spans both coding and noncoding regions, with emphasis placed on well-known genes, promoters, miRNAs, and telomeric regions. Probe design and selection have been carefully optimized and validated for maximal sensitivity and specificity. In this study, therefore, we examined high-resolution array-based CGH for the evaluation of genetic alterations caused by BPA exposure.

Y Chromosome Deletion Detection

To determine whether Y chromosome abnormalities

Table 1. Biochemical data on the BPA-treated mice.

	Control	BPA
Terminal body weight (g)	44.39±2.12	45.32±1.67
Testis weight (%)	0.35±0.03	0.28±0.06
Epididymis weight (%)	0.14±0.02	0.12±0.02
Sperm motility (%)	76.25±14.48	82.33±9.37
Sperm counts ($\times 10^6$)	7.3±2.4	5.0±1.0

The result are expressed as the mean±SD.

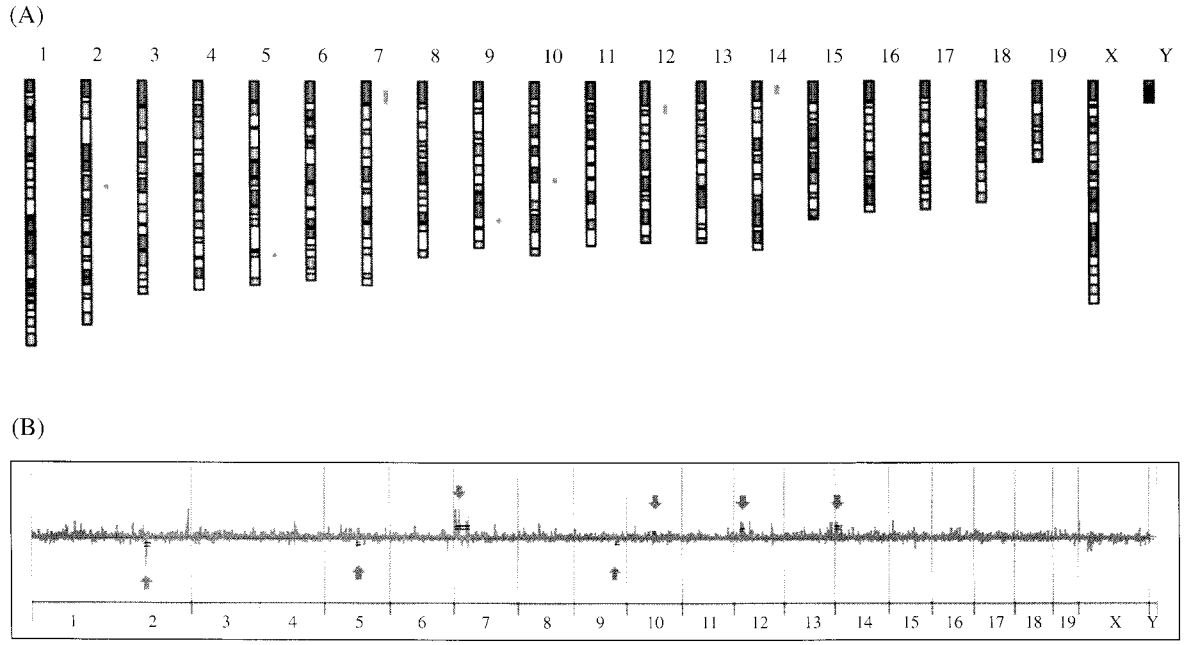

Figure 2. Location of genomic alteration in total genome view. (A) Location of gain or loss in DNA copy number. Red color represents a gain, green is a loss. (B) Multi-panel view. Arrow indicates a region of gain or loss.

were associated with infertility in males after BPA-treatment, we performed a Y chromosome-based microdeletion detection technique in a BPA-treated *in vitro* system. The sample was duplicated for the reliability of the data. PCR was performed under the recommended conditions. As a result of multiplex PCR, as is shown in Figure 1, all 32 probes were detected. These results showed no deletion on the Y chromosome as the result of BPA-treatment. That is to say, BPA treatment did not directly affect reproductive system-associated aberrations of the Y chromosome, such as infertility.

Morphological Changes of BPA-treated Mice

To collect and evaluate the morphological toxicology data, we determined the weights of the body, the

Table 2. List of genomic altered-region.

Chromosome	Cytoband	#Probes	Amp/Del	P-value
Chr2:77739470-77848433	qC3	5	−2.337	7.54e-24
Chr5:128329169-128329869	qG1.2	1	−2.573	1.86e-15
Chr7:7312289-10272836	qA1-qA2	21	1.028	3.69e-10
Chr9:101951419-101951819	qF1	1	−3.301	7.01e-10
Chr10:72178606-72179006	qB5.3	1	2.038	7.19e-09
Chr12:17803411-25366481	qA1.2-qA1.3	99	0.341	2.41e-14
Chr14:4551029-10397399	qA1	105	0.493	2.93e-12

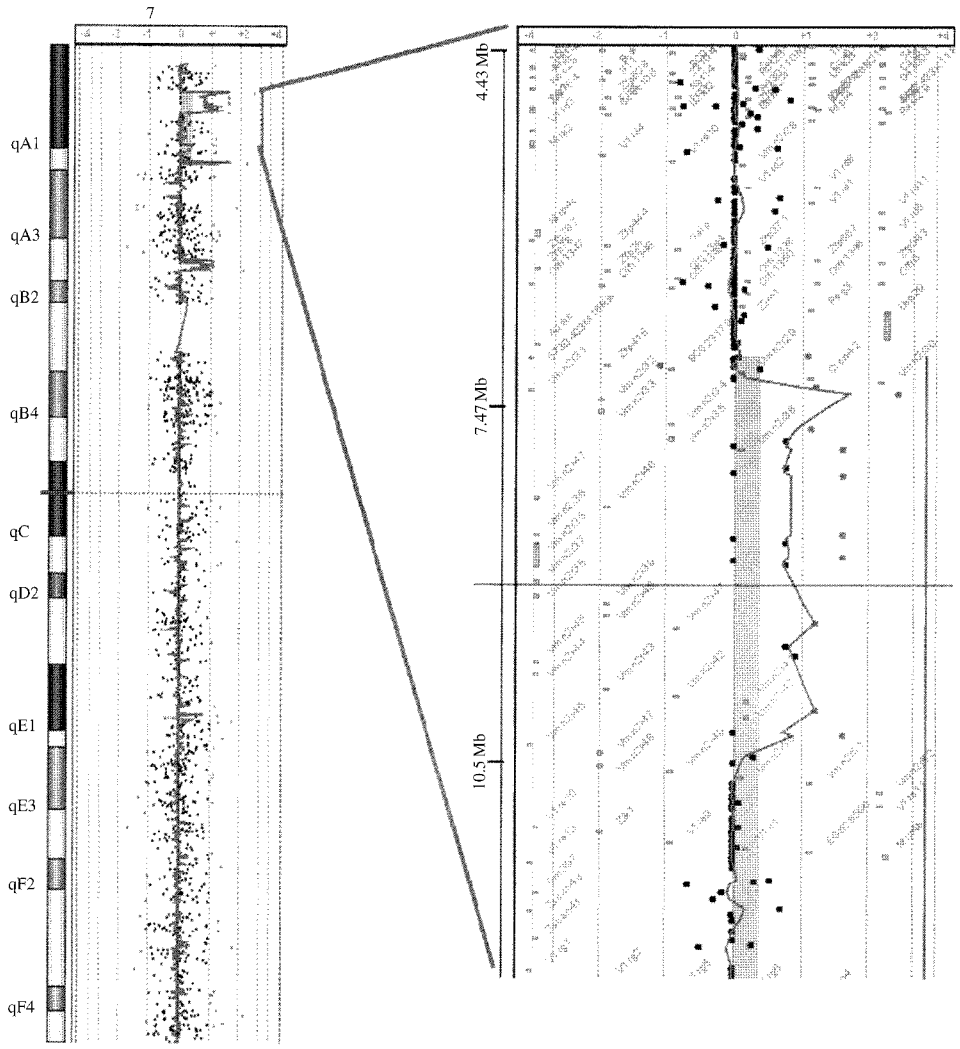

Figure 3. Profile of chromosome 7 total and qA1-qA2 region. Zoom in altered region and associated gene, red point is gain probe, black point is normal probe, green point is loss probe. Peak is a region of DNA copy number variation. Many vonomesal receptor subfamily genes are included in chr7 qA1-qA2.

testis, and the epididymis. The body weights of the BPA-treated mice evidenced no specific changes, and the testis and epididymis evidenced minimal changes in weight. These results show that the BPA-treated mice did not undergo morphological changes. However, sperm counts in the BPA-treated mice decreased as compared to the controls (Table1). Although no morphological weight changes were noted, we determined that BPA-treatment causes changes in the sperm count, and thus we associated BPA directly with the male reproductive system.

Genomic Variation

To assess the genomic alterations in the mice testes as a result of BPA exposure, we utilized oligonucleotide array-based CGH, with an Agilent mouse genome CGH 105K array. The Agilent CGH array enables the detection of genomic alterations with a resolution of 10 kb in the BPA-treated mice. For data analysis, we used Genomic Workbench Standard Edition 5.0.14 software (Agilent Technologies, CA). Following data extraction and normalization, we selected the region of copy number variation using the ADM2 finding method. A total of seven features were detected in the regions of DNA copy number variation in the BPA-treated mice (Table 2, Figure 2). Four regions evidenced increases (gain) and three regions evidenced reductions (loss) in DNA copy numbers. Chr7, 10, 12, and 14 showed a small gain, and chr2, 5, and 9 showed a small loss. Some loss-region of the DNA copy number were quite small, and one probe region was approximately 400-700 bp. Some of the gain-regions were substantially larger than the loss-regions. Genes within the genomically alternating regions were annotated using a mouse genome database (UCSC mm8, NCBI Build 36). Interestingly, the increased region of chr7:7312289-10272836 (the average of log2 ratio in these segments is 1.028) includes many genes of vomeronasal receptor subfamilies (Figure 3). The vomeronasal receptor was associated with pheromone response and signal transduction.

Functional Analysis

We also investigated the functions genes associated with regions of genomic alteration. For functional analysis, we utilized GeneSpring GX 10.0.1 (Agilent Technologies, CA), ArrayToGO, and ArrayToKegg (GenoCheck, Ansan, Korea) software[9]. Genomic alteration-related genes are classified as GO ontology-related (Figure 4). As a classification, many genes are associated with pheromone response (Vomeronasal 1 receptor, J2, E11, L1, G2, G3, G4, G5, G6, G7, G11, K1) and signal transduction (Grlf1, Hif3a, Gpr77, C5ar1, Npas1, Gng8, Ptgir, Ywhaq). Interestingly, genes associated with pheromone response are located in the chr7-altered region. Other related functions include transcription (Zfp128, Trim28, Npas1), protein transport (Napa, Chmp2a, Ap2s1) and lipid metabolic processes (Slc27a5, Sult2a1, Acox2)

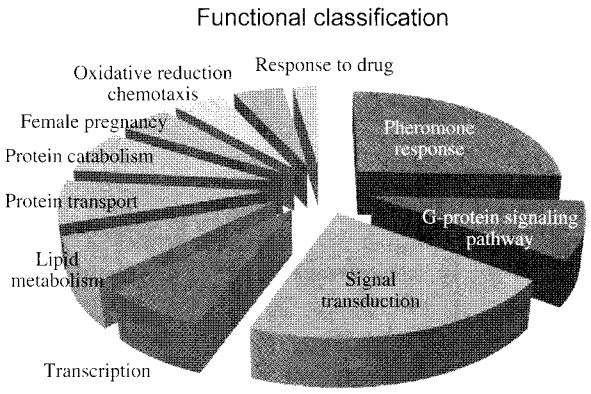

Figure 4. Functional classification of genomic alteration-related genes. GO ontology was analyzed by GeneSpring software. Pheromone response-related genes and signal transduction-related genes are substantially included in the region of genomic alteration by BPA treatment.

Discussion

BPA is broadly available in households, industry, and all around, but is also associated with toxicity, particularly endocrine disruption. In many previous studies, BPA causes reproductive toxicity in male rodent systems. However, the mechanisms underlying these male reproductive toxic effects have yet to be clarified[10].

To elucidate Y chromosome abnormality as the result of BPA-treatment, we attempted to detect Y chromosome microdeletions in an *in vitro* system. We detected no changes in the Y chromosome. As a result, BPA treatment did not directly affect the infertility induced by Y chromosome abnormality.

Oligonucleotide array-based CGH experiments are a powerful tool that can be used to detect genomic alterations, such as gain or loss of DNA copy numbers. In this study, we assessed variations in the DNA copy number by BPA-treatment using array-based CGH. We determined that some DNA copy numbers changed after BPA treatment-four regions increased and three regions decreased. Interestingly, the Chr7:qA1-qA2 of the increased region includes many genes that are associated with pheromone response. Pheromones, which are endogenous hormones, induce a chemical stimulus that elicits a natural response in another individual, affecting either the behavior or physiology of

the other. In males, pheromones that transmit information about genotype are secret[11-13]. According to our results, abnormality in these pheromone responses may directly or indirectly cause disorders of the reproductive system in males.

In conclusion, although our study did not clearly demonstrate the mechanisms relevant to toxicity in the male reproductive system, we know that BPA causes variations in DNA copy numbers in specific regions, most notably an abundance of pheromone receptors. This study may help us to understand toxicogenomics approaches utilizing array-based CGH.

Materials & Methods

Animals and Chemicals

Approximately 10-week-old Crj : CD-1(ICR) male mice (SLC, Japan) were maintained under a 12-h light /dark cycle in an animal room at a controlled temperature and humidity for 2 weeks prior to the experiment. The mice were provided with standard food pellets and water *ad libitum*. BPA was obtained from Sigma Aldrich Chemicals (USA). BPA dissolved in dimethyl sulfoxide (DMSO)/corn oil (0.5 : 9.5, v/v) was injected subcutaneously at a concentration of 20 mg/kg body weight. The chemicals were administered every other day for 30 days. The control mice were given corresponding quantities of the vehicle. The mice were sacrificed after 30 days and were carefully dissected in order to count the sperm and collect tissue samples. Five or six animals were used in each experiment. Relative organ weight was calculated as the ratio of the right organ weight to body weight. Epididymal sperm were collected by chopping the cauda epididymis in 1 mL of CO_2-independent medium (pH 7.4-7.6, Invitrogen, USA) with 5 mg/mL bovine serum albumin, and the sperm were incubated for 5 min at 37°C. Approximately 10 μL of this solution was placed on a glass slide set on a warming plate, and motile and non-motile sperm were counted (this method is the same as in our previous study, Oh *et al.*[14]).

Y Chromosome Deletion

Genomic DNA was extracted from BPA-treated human cells using DNase mini kits (Qiagen, USA) in accordance with the manufacturer's instructions. The concentration of each total genomic DNA was quantified using NanoDrop (NanoDrop Technologies, Wilmington, DE, USA) and its quality was verified via 1% agarose gel electrophoresis. The detection of Y chromosome abnormalities was conducted using a Y chromosome microdeletion detection kit (LG Life Sciences, Korea). The Y chromosome microdeletion detection kit is designed to detect deletions occurring in Yq AZF, and consists of STS markers. This system comprises 32 primer pairs that have been combined into 4 sets for use in multiplex PCR and 4 sets consisting of 15 STS markers and 1 internal control. We conducted PCR in accordance with the manufacturer's instructions. The results were assessed via 3% agarose gel electrophoresis.

Array-based CGH

Genomic DNA was extracted from the mice testis treated with BPA using a DNase mini kit (Qiagen, USA) in accordance with the manufacturer's instructions. The concentration of each total genomic DNA was quantified with NanoDrop (NanoDrop Technologies, Wilmington, DE, USA) and its quality was assessed via 1% agarose gel electrophoresis.

Each genomic DNA (3 μg) was digested to 200-1,000 bp DNA with the DpnII (New England Biolabs, Beverly, MA, USA) enzyme. The fragmented DNA was then labeled with Cy3-dCTP or Cy5-dCTP (Amersham Bioscience, UK) using the Bioprime DNA Labeling System (Invitrogen Life Technologies, Carlsbad, CA, USA). The labeled DNA mixture was concentrated with purification columns. The concentrated Cy3 and Cy5 labeled DNA were resuspended in 30 μL of hybridization solution (GenoCheck, Korea). After the two labeled DNAs were mixed, they were placed on the Agilent Mouse genome CGH 105K array (Agilent Technologies, Inc., CA) and covered with a MAUI DC chamber (Biomicro Systems, Inc. UT). The slides were then hybridized for 72 h at 65°C in a MAUI system (Biomicro Systems, Inc. UT). The hybridized slides were washed in 2xSSC, 0.1% SDS for 2 min, 1xSSc for 3 min, and then 0.2xSSC for 2 min at room temperature. The slides were centrifuged at 3,000 rpm for 20 sec to dry.

Data Analysis

We performed quadruplicate trials to verify the reliability of array-data by individual variation. Data was extracted to normalized log2 (test/ref) ratio data using Feature Extraction 9.5.3.1 (Agilent Technologies, Inc., CA). To select the copy number variation, we used Agilent Genomic Workbench Standard Edition 5.0.14 software (Agilent Technologies, Inc., CA). We also the used ADM-2 (aberration detection module-2) finding method, threshold: 5.0, to select of copy number variation, and used GEAR (Genomic Enrichment Analysis of Regional DNA copy number changes) as a statistical method (hypergeometric distribution, uncorrected P-values < 0.02)[15]. ArrayToKegg software (GenoCheck, Ansan, Korea) was utilized for the functional analysis of genes within the copy number changed

region (DNA).

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