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Effects of benzo[a]pyrene on mouse germ cells: heritable DNA mutation, testicular cell hypomethylation and their interaction with nucleotide excision repair.

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Abstract

Paternal exposure to the environmental contaminant benzo[a]pyrene (B[a]P) may pose a genetic risk to future generations, because B[a]P or its metabolites can reach the testis and cause DNA damage and global hypomethylation. DNA damage and global hypomethylation can promote genomic instability in male germ cells resulting in mutations that are subsequently transmittable to offspring, especially when DNA repair is compromised. To study the ability of B[a]P to cause mutations in male germ cells and alterations in DNA methylation in testicular cells, and to investigate the relationship between these endpoints, we orally treated DNA repair deficient *Xpc*$^{-/-}$ and DNA repair proficient *Xpc*$^{+/+}$ male mice with B[a]P or vehicle for a period of 6 weeks (13 mg/kg bw, 3x per week). Mice were crossed with untreated female mice 6 weeks after the last exposure to ensure the analysis of mutations originating from spermatogonial cells. Offspring tissues were collected on post-natal days 21-28. In the exposed fathers, B[a]P related DNA adducts were found in testes, indicating that the exposure reached the gonads. DNA adduct levels were similar in *Xpc*$^{-/-}$ and *Xpc*$^{+/+}$ treated fathers. Global methylation of retrotransposable elements (LINE-1, SINE-B1 and SINE-B2) was significantly decreased in testis DNA from B[a]P treated fathers, irrespective of DNA repair genotype. Mutation rate at *Ms6-hm* and *Hm-2* tandem repeat loci was only increased in offspring of B[a]P exposed *Xpc*$^{-/-}$ fathers. Mutation rates rose from 0.017 to 0.056 in offspring from control and treated *Xpc*$^{-/-}$ fathers, respectively (P=0.034), whereas mutation rates were 0.028 and 0.029 in offspring from control and exposed DNA repair proficient fathers. The results indicate that B[a]P is able to induce hypomethylation in testicular DNA, and causes heritable mutations in offspring from *Xpc* deficient male mice. Potential health effects of paternal B[a]P exposure in offspring warrants extensive further investigation.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are important occupational and environmental pollutants. PAH potentially pose a genetic risk to the human population because their metabolites can reach germ cells and cause DNA damage[1]. Exposure to complex mixtures of contaminated air that contain PAH has indeed been associated with the induction of paternally transmitted germ line mutations in free living herring gulls (*Larus argentatus*) nesting near steel mills[2] and in laboratory mice caged in similar locations[3,4]. It is unclear to what extent the presence of PAH in those exposures contributes to the observed increases in germ line mutations. Exposure to PAH is unavoidable and it is therefore important to definitively establish whether PAH cause germ line mutations and to evaluate potential health risks for the offspring. Previous results using the classical specific locus test for visible markers were negative or inconclusive for heritable point mutations in benzo[a]pyrene (B[a]P) exposed male mice[5]. However, dominant lethal mutations were induced in B[a]P-exposed post-meiotic male germ cells[6]. In addition, we recently demonstrated that B[a]P causes *lacZ* gene mutations in male mouse pre-meiotic germ cells following six week exposures[7]. However, it still remains to be determined whether these mutations are actually transmitted to the offspring.

The mutagenicity of many PAH, including the well-studied and widespread B[a]P, is usually explained by their conversion to DNA reactive derivatives that can directly damage DNA to form pro-mutagenic DNA lesions. More recently, it has become clear that B[a]P can also cause 'indirect' DNA damage, because B[a]P exposure leads to global DNA hypomethylation[8]. Altered methylation of certain DNA regions has been associated with genomic instability[9]. In addition, exposure to other mutagens (*e.g.*, radiation) has been shown to impact testicular methylation and methylation patterns in the unexposed descendants of mutagen-exposed males, suggesting potentially heritable effects[10]. Epigenetic endpoints are not presently evaluated in the context of germ line genetic risk assessment, and it is important that this major gap is addressed.

We have previously shown that altered methylation patterns are potentially involved in increased germ line mutations in the offspring of air pollution exposed fathers[11]. Germ line mutations in offspring were determined in expanded simple tandem repeats (ESTR), which consist of 4 to 6 bp repeat units in long tandem arrays. ESTRs are unstable in the germ line and tend to mutate by insertion or deletion of a number of repeat units[2,3,11]. Studies analyzing ESTRs have established that chemical mutagens[12], radiation[13] and air pollution[2,3] induce germ line mutations in mice. These mutations are identified as novel alleles present in the DNA fingerprints of offspring that can not be ascribed to either parent. It has been suggested that chemicals that modify chromatin conformation through changes in DNA methylation may result in ESTR mutations[14]; hypomethylation in the proximity of tandem repeat loci may lead to the

formation of secondary DNA structures that are more prone for replication errors[15]. On the other hand, hypermethylation may compromise the ability of DNA repair enzymes to access and repair DNA or hypermethylation of specific DNA repair genes may lower DNA repair activity[9]. The exact role of DNA methylation in ESTR mutagenesis is currently unknown. Therefore, it is important to understand how the combination of DNA damage and changes in global DNA methylation can promote genomic instability in male germ cells resulting in mutations that are subsequently transmittable to offspring.

In the present study we investigate the ability of B[a]P to cause mutations in endogenous ESTR sequences (*Ms6-hm* and *Hm-2*) in the male mouse germ line that are transmitted to offspring. We also analyzed the methylation status of the retrotransposons LINE-1, SINE-B1 and SINE-B2 in testis DNA after paternal exposure to B[a]P. To this end, male mice (C57BL/6) deficient for the nucleotide excision repair (NER) gene *Xpc* (*Xpc*$^{-/-}$) or their wild type (wt) counterparts were exposed to B[a]P for 6 weeks and were crossed with female mice (Balb/C) 6 weeks after the final exposure. The *Xpc*$^{-/-}$ mouse model was included in this study because it is a more sensitive model for mutation induction than its wild type counterpart for somatic cells[16], and thus may increase our probability of detecting significant effects on germ line mutation rates.

Materials and methods

Animal and exposure

Mice were bred and maintained under pathogen-free conditions in a 12-hr light-dark cycle at the animal facilities of the Netherlands Vaccine Institute (NVI, Bilthoven, the Netherlands) and received food and water *ad libitum*. Experiments were approved by the Institute's Animal Ethics Committee and were carried out according to their guidelines. DNA repair deficient *Xpc*$^{-/-}$ male mice or wild type (wt) male littermates (C57BL/6) were subchronically exposed for 6 weeks to B[a]P (3 times per week, 13 mg/kg bw dissolved in sunflower oil) by oral gavage. Control male mice received sunflower oil 3 times per week over the 6 weeks. All male mice (6 mice per group) were subsequently crossed with 2 unexposed Balb/C wt female mice 6 weeks after the last exposure in order to obtain offspring. This 6 week break ensured that the mutations detected originated from spermatogonial stem cells and dividing spermatogonia (note that it takes approx. 6 weeks for sperm cells to reach the epididymides/ ejaculate after the first mitotic division of a spermatogonial stem cell). Male mice were euthanized after successful fertilization, while female and offspring mice were euthanized approximately 3-4 weeks after birth of offspring mice. Testes and tail were collected from the fathers, and livers and tails were collected from the mothers and offspring mice; tissues were snap frozen in liquid nitrogen and stored at -80°C.

DNA isolation

Mouse tails were cut into small pieces and organs (testes and liver) frozen in liquid nitrogen were crushed. Two ml lysis buffer (50 mM NaCl, 18.75 mM EDTA, 15 mM TrisHCl pH 8.5 and 0.5% SDS) and 30 µl proteinase K [25 mg/ml] were added. Samples were then incubated in a shaking water bath at 56°C overnight. Genomic DNA was extracted sequentially with phenol: chloroform: isoamylalcohol (25:24:1, v/v/v) and chloroform: isoamylalcohol (24:1, v/v). DNA was precipitated with 2 volumes of cold 100% ethanol and 10% 3M NaAc (pH 5.2). Pelleted DNA was washed with 70% ethanol, dried under nitrogen and dissolved in mQ-water. Concentrations and purity of all samples were confirmed by spectrophotometry.

B[a]P-DNA adducts in testis

DNA adducts were measured in testes of B[*a*]P-exposed wt and *Xpc*$^{-/-}$ mice euthanized 6 weeks after the final exposure. To determine BPDE-DNA adducts by ^{32}P-postlabeling [17], 10 µg DNA was digested using micrococcal endonuclease (0.57 units per sample, Sigma-Aldrich, Zwijndrecht, The Netherlands) and spleen phosphodiesterase (4.75 micrograms per sample, Sigma-Aldrich) for 4 h at 37°C. Subsequently, samples were treated with nuclease P1 (3.25 micrograms per sample, MP Biomedicals, Eindhoven, The Netherlands) for 30 min at 37°C in a total volume of 12.5 µl. The largest fraction of the modified nucleotides (10.5 µl) was labeled with [γ-^{32}P]-ATP (50 microcurie per sample, MP Biomedicals) by incubation with T4-polynucleotide kinase (11.5 units per sample, Fermentas, St Leon-Rot, Germany) for 30 min at 37°C. Radiolabeled adduct nucleotide bisphosphates were separated by thin-layer chromatography on polyethyleneimine cellulose sheets (Macherey-Nagel, Düren, Germany) in the following solvents—D1: 1 M NaH_2PO_4, pH 6.5; D2: 8.5 M urea and 5.3 M lithium formate, pH 3.5; and D3: 1.2 M lithium chloride, 0.5 M Tris, and 8.5 M urea, pH 8.0. In each experiment, three standards of [^{3}H]benzo[a]pyrene-dihydrodiol-epoxide modified DNA with known modification levels (1 per 10^7, 10^8, and 10^9 nt) were run in parallel for quantification purposes and mQ-water was used as a negative control. DNA adduct levels were quantified using the FujiFilm imaging system FLA-3000, BASReader software, and Advanced Image Data Analyzer software (all from Raytest, Tilburg, The Netherlands), with a detection limit of < 1 adduct per 10^9 nt per individual DNA adduct spot. A small fraction of the digest (2 µl) was diluted in 36.3 µl of mQ-water and was used to determine the amount of DNA in the assay. Tris-HCl (pH 7.4) and alkaline phosphatase (0.01 U/µl, Sigma-Aldrich) were added to the samples after which samples were incubated at 37°C for 45 min. Nucleosides were subsequently separated and detected by reversed phase HPLC-UV detection using 10% aqueous methanol containing 94 mM KH_2PO_4, 13 mM K_2HPO_4, 26 mM KCl, and 0.5 mM EDTA as mobile phase. The peak area of deoxy-guanine (dG) was used to correct for differences in DNA content between samples.

Analysis of retrotransposon methylation in fathers' testis and offspring liver

Analysis of the methylation pattern of the repetitive elements (i.e., long interspersed nucleotide elements (LINEs) and short interspersed nucleotide elements (SINEs)) was performed using the methylation-sensitive McrBC real-time PCR assay[18]. One microgram of genomic DNA was digested overnight at 37°C using 10 U of McrBC (New England Biolabs, Beverly, MA, USA), an endonuclease that cleaves DNA containing 5-methylcytosine but will not cleave unmethylated DNA. Digestion was checked in several randomly selected samples using gel-electrophoresis. The DNA strand breaks prevent amplification of methylated DNA in the quantitative real-time PCR assay. A 2-step quantitative real-time PCR was performed using IQ SYBR Green Supermix (Bio-Rad Laboratories, Veenendaal, The Netherlands) with 4 ng of McrBC-digested DNA and 25 pmol of each primer (Eurogentec, Maastricht, The Netherlands) in a reaction volume of 25 µl. The forward and reverse primer sequences for the different repetitive elements and endogenous reference were as follows: LINE1-ORF2, 5′-TTTGGGACACAATGAAAGCA-3′ and 5′-CTGCCGTCTACTCCTCTTGG-3′; SINEB1, 5′-GTGGCGCACGCCTTTAATC-3′ and 5′-GACAGGGTTTCTCTGTGTAG-3′; SINEB2, 5′-GAGATGGCTCAGTGGTTAAG-3′ and 5′-CTGTCTTCAGACACTCCAG-3'. The cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 45 s and 58°C for 90s using an iCycler (Bio-Rad Laboratories). Data were analyzed using the MyiQ software system (Bio-Rad Laboratories). Ct values were normalized using an endogenous reference gene (*Hprt*) and compared with controls (i.e., average Ct value of unexposed control samples and expressed as relative expression ($2^{-\Delta\Delta Ct}$)). An increase in PCR amplification products is indicative of hypomethylation, whereas a decrease in PCR amplification products indicates hypermethylation.

ESTR mutation analysis in offspring

Tail DNA (12 µg) was digested overnight at 37°C to completion with *AluI* restriction enzyme (New England Biolabs), and digestions were checked for completion on an agarose gel. DNA was size-fractionated in 40 cm long 0.8% agarose gels (120V) until the 2 kb size marker had just run off the gel, and DNA was transferred to a nylon membrane (Hybond-XL, Amersham Pharmacia) by Southern blotting. DNA fingerprints were generated by sequential hybridization with ^{32}P-labeled synthetic ESTR probes *Ms6-hm* [19] and *Hm-2* [20], and were visualized by using FujiFilm imaging system FLA-3000 (Raytest). Blots were completely stripped of probe DNA between hybridizations by boiling in 0.1% SDS and shaking for 2 minutes. All samples were run with 10 ng of 1 kb ladder as size standard. Fingerprint bands in offspring that deviated from the parental progenitor alleles by at least 1 mm were scored as mutations. Scoring was performed without knowledge of exposure regimen and verified by a second observer.

Statistics

Data are presented as average ± standard error of the mean. Changes in methylation of transposable elements and litter sizes were statistically evaluated using non-parametric Kruskal-Wallis tests. ESTR mutation rates were calculated as the number of mutant bands out of the total scored and compared using a modified two-tailed Fisher's exact test. Mutation rates are presented with 95% confidence intervals for the *Ms6-hm* and *Hm-2* data combined. A difference was considered significant at $p < 0.05$.

Results

DNA damage in testis and litter sizes.

B[a]P related DNA adducts after 6 weeks of exposure and 6 weeks of recovery were detected in all testis samples of exposed fathers, but no statistically significant difference was observed between *Xpc*$^{-/-}$ mice (2.2 ± 0.5 adducts per 10^8 nt) and wt animals (1.6 ± 0.3 adducts per 10^8 nt). No DNA adducts were detected in testis of unexposed animals. These data indicate that B[a]P or its metabolites reached the testes and caused potentially promutagenic DNA lesions in both *Xpc*$^{-/-}$ and wt mice.

Twelve *Xpc*$^{-/-}$ breeding pairs were established in both the B[a]P exposed and control group, of which ten pairs successfully produced offspring in each group. Litter sizes of successful pairs showed a significant difference between B[a]P exposed and control groups, with significantly smaller litter sizes in the B[a]P exposed groups (8.5 ± 1.5 *vs*. 6.1 ± 2.3, P = 0.014, for control and BaP treatment groups, respectively). Litter sizes were also smaller after B[a]P exposure in wt animals, but this did not reach statistical significance (6.8 ± 2.4 *vs*. 5.4 ± 1.8 in the unexposed and exposed group, respectively).

Hypomethylation of testis DNA in B[a]P exposed animals and liver of offspring.

Testicular DNA of B[a]P exposed fathers was hypomethylated at the *LINE-1, SINE-B1* and *SINE-B2* locus compared to unexposed controls (Table 1). Although this hypomethylation was more pronounced in exposed wt animals, it was also observed in *Xpc*$^{-/-}$ mice after B[a]P exposure. However, this result only reached statistical significance in *Xpc*$^{-/-}$ mice when all three loci were analyzed together (P=0.002). Global methylation of the retrotransposons in liver samples of offspring was not different for offspring of exposed and unexposed fathers (Table 1).

Mutation rates in *Ms6-hm* and *Hm-2* ESTR loci after B[a]P exposure.

Mutation rates were determined in the offspring of B[a]P exposed and control fathers by analyzing the DNA fingerprints of *Ms6-hm* and *Hm-2* ESTR loci of parents compared to their

offspring. An example of a mutant band detected with the ESTR single-locus probe *Ms6-hm* is shown in Figure 1. Mutation rates of paternal origin were elevated in the offspring of the B[a]P exposed group compared to the control group, but only if DNA repair was not effective due to *Xpc*-knock out (0.056±0.020 and 0.017±0.013, respectively, P=0.034, Table 2). B[a]P exposure did not cause a significant increase in the number of mutations transmitted to offspring in DNA repair proficient mice (0.029±0.018 *vs*. 0.028±0.017 for offspring of exposed and unexposed fathers, respectively). Mutation rates were higher in the wt control group than in the *Xpc*-/- control group (0.028 and 0.017, respectively), but this difference did not reach statistical significance. When both control groups were combined, the effect of B[a]P in *Xpc*-/- mice remains statistically significant (P=0.013).

Discussion

We have little understanding of the mechanisms underlying genomic instability that contribute to *de novo* genetic disease and chromosomal abnormalities in human live births[21]. Recent studies suggest that lifestyle, such as smoking, could enhance heritable mutations and genetic instability[22]. We hypothesize that these effects may be mediated via direct DNA damage to the germ line genome, and also via altered DNA methylation. To better understand heritable genomic instability, it is important to define how genome methylation in the germ line of exposed parents can affect the genome of their progeny, in combination with other well-known protective mechanisms, such as DNA repair. It has been proposed that DNA methylation at CpG sequences is a major defense mechanism against genetic instability. Cigarette smoke and environmental contaminants are complex mixtures that contain PAH and that have previously been associated with the induction of heritable gene mutations[22,23]. In the present study, we show that the model PAH, benzo[a]pyrene, is indeed able to induce genomic alterations in ESTRs that were subsequently inherited by the offspring, but only in nucleotide excision repair deficient mice (*Xpc*$^{-/-}$). In contrast, global hypomethylation of testis DNA was predominantly found in wt animals, which does not correspond to the absence of B[a]P induced ESTR mutations in these animals. We note that testicular DNA represents a mixture of both somatic and germ cells. However, with the assumption that changes in DNA methylation of testicular DNA reflect changes in germ cell DNA methylation, the data do not support that B[a]P induced ESTR instability depends solely on genome hypomethylation.

The present data build further on previous experiments[7] in which B[a]P exposure was found to increase *LacZ* mutant frequencies in the sperm of wt mice after exposure to B[a]P, while no effect was observed in *Xpc*$^{-/-}$ mice. This result is the opposite of what was found for ESTR mutations in the current study. We note that mutations detected in *LacZ* and ESTR originate

from very different underlying molecular mechanisms. Direct lesions (*e.g.*, DNA adducts) in the *LacZ* DNA sequence cause mutations at this locus. In contrast, ESTR mutations result from indirect mechanisms that are thought to be caused by cell cycle arrest, the subsequent formation of secondary structures at replicating ESTR loci during this arrest, and polymerase errors at these secondary structures upon replication re-initiation[24]. Spontaneous ESTR mutations were previously reported to be increased in *Xpc*$^{-/-}$ mice compared to their wt-counterparts[25]. We were unable to reproduce the higher spontaneous ESTR mutation frequency in *Xpc*$^{-/-}$ mice, but in our study *Xpc*$^{-/-}$ animals were more sensitive to the induction of ESTR mutations by B[a]P. These findings indicate that nucleotide excision repair is involved in preventing bulky DNA adduct-induced repeat mutations. Although there were no differences in adduct levels in the two groups, these measures were taken in mature sperm and not spermatogonia. It is possible that increased adducts present in spermatogonia resulted in increased incidence of cell cycle arrest and increase opportunities for polymerase errors in *Xpc-/-* mice. Indeed, the decreased litter size observed in the *Xpc-/-* strain suggests that more cells are recognized as damaged and undergo apoptosis in *Xpc-/-* mice. This finding is consistent with B[a]P induced problems in fertilization[26], apoptosis primarily in spermatogonia [27,28] and the previous dominant lethal test results[6]. An additional noteworthy difference in these studies is also the measurement of *LacZ* mutations in sperm *versus* the measurement of repeat mutations in the offspring. We cannot at this time eliminate the possibility that some ESTR mutations may have arisen in early embryogenesis. Indeed, our data indicate that bulky DNA adducts were present in the mature sperm at the time of fertilization and thus the embryo 'inherited' damaged DNA.

Interestingly, NER has also been implicated in active DNA demethylation[29], which seems to correspond to the more extensive demethylation of testis DNA after exposure to B[a]P in our NER proficient animals. If ESTR mutations are dependent on methylation status, one would thus expect a higher ESTR mutation rate in pups of exposed wt animals. However, we found the opposite here. It should be noted that *Xpc* has been linked to functions outside of NER, since *Xpc* deficient mice show divergent tumor spectra when compared to other NER deficient mouse models[30]. Both *in vivo* and *in vitro* experiments indicate that *Xpc* appears to be involved in the initiation of several DNA damage-induced cellular responses, including removal of oxidative DNA damage, redox homeostasis and cell cycle control [31]. Therefore, based on the above knowledge and our data, we hypothesize that a more open chromatin structure near repetitive sequences caused by hypomethylation, in combination with these additional mechanistic roles of Xpc, led to increased susceptibility for ESTR mutation induction in the germ cells or early embryo of *Xpc*$^{-/-}$ mice. As noted, we measured global demethylation in whole testis DNA. It can be argued that this does not measure methylation changes occurring in spermatogonia at the time that mutations arose, or the mature sperm at the time of mating. Methylation may be cell type specific and transient, thus changes that occurred in spermatogonia may not be

adequately reflected in measures taken on whole testis at the time of sampling in this study [32]. Moreover, we previously showed that environmentally polluted air that contained PAH actually hypermethylated sperm DNA[11]. On the other hand, it has been reported that B[a]P predominantly leads to global hypomethylation[33], so the effects observed following exposure to contaminated air may not be PAH-dependent. Therefore, the lack of correlation shown here must be interpreted with caution, and further studies are needed to unravel the link between DNA methylation and ESTR mutations.

We studied global DNA methylation by focusing on retrotransposons that form a large part of the murine genome; cytosine methylation of long interspersed nuclear elements (LINE1) and short interspersed nuclear elements (SINE B1 and B2) is known to play an important role in transcriptional repression of these retrotransposons[34]. Hypomethylation may result in activation of these retrotransposons, which copy themselves to RNA and then back to DNA that is subsequently integrated into the genome. Indeed, it has been found that B[a]P is able to activate retrotransposons *in vitro* [35]. Thus, BaP exposure causes the loss of an important defense mechanism against the activation of transposable elements in testicular DNA. Retrotransposons insert into the genome without apparent site specificity, and de *novo* insertions of transposable elements occasionally result in disease. The first description of a transposable element causing a disease in humans was reported in 1988; a LINE1 insertion into the coagulation factor VIII gene caused hemophilia A in 2 out of 240 unrelated patients[36]. Since that time, many *de novo* transposon insertions that cause disease or alter disease risk in humans and mice have been reported; for example, several types of cancer (including leukemia and breast cancer), hypo-betalipoproteinemia, insulin-independent diabetes and glycogen storage disease[36]. Retrotransposon insertion cannot only disrupt gene structures; it can also affect gene expression by altering transcription of nearby genes. Interestingly, retrotransposons may be particularly active during spermatogenesis or oogenesis, but the integration events usually occur during embryogenesis rather than in germ cells and are probably not heritable [37]. Indeed, the hypomethylation of these abundant DNA sequences measured in testicular cells was not transmitted to the offspring, since we did not see hypomethylation in liver DNA of offspring animals. Interestingly, Filkowski *et al*. [10] showed that paternal exposure to radiation altered methylation in offspring thymus tissue. Transmittable effects may be tissue- or exposure-specific; this area of research needs significant further attention. It would also be worthwhile to focus on gene-specific alterations instead of global alterations in methylation, because methylation of specific genes may have different probabilities of 'surviving' the reprogramming of the epigenome after fertilization than retrotransposons.

A recent study suggested that cigarette smoking is a germ cell 'mutagen' [22]. B[a]P is a well-known constituent of cigarette smoke, and in the current study, we show that B[a]P is indeed capable of inducing heritable gene mutations that are transmitted to the offspring. Of course

cigarette smoke contains many more compounds that may reach germ cells and cause DNA damage. Indeed, cigarette smokers have increased levels of oxidative DNA damage [38], DNA strand breaks[39], bulky DNA adducts[40], and chromosomal abnormalities[41] in their sperm. Our work demonstrates that BaP can cause mutations in spermatogonia that are transmitted to offspring, and suggests that BaP may be one of the agents responsible for the genotoxicity of tobacco smoke to germ cells. Whether or not this is through direct interaction of B[a]P derived metabolites with DNA, or through indirect effects of B[a]P on DNA methylation profiles remains to be determined. However, our data suggest an interaction of B[a]P-induced heritable genetic effects and epigenetic effects in gonadal tissues with nucleotide excision repair. Future studies should elucidate whether B[a]P induced mutations in offspring also increases health risks.

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References

1. N. Verhofstad, C. T. M. van Oostrom, J. van Benthem, F. J. van Schooten, H. van Steeg, and R. W. L. Godschalk, *Environ. Mol. Mutagen.*, 2010, **51**, 123–9.
2. C. L. Yauk and J. S. Quinn, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 12137–41.
3. C. M. Somers, C. L. Yauk, P. A. White, C. L. J. Parfett, and J. S. Quinn, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 15904–7.
4. C. M. Somers, B. E. McCarry, F. Malek, and J. S. Quinn, *Science*, 2004, **304**, 1008–10.
5. L. B. Russell, P. B. Selby, E. von Halle, W. Sheridan, and L. Valcovic, *Mutat. Res.*, 1981, **86**, 329–54.
6. W. M. Generoso, K. T. Cain, C. S. Hellwig, and N. L. Cacheiro, *Mutat. Res.*, 1982, **94**, 155–63.

7. N. Verhofstad, C. T. M. van Oostrom, E. Zwart, L. M. Maas, J. van Benthem, F. J. van Schooten, H. van Steeg, and R. W. L. Godschalk, *Toxicol. Sci.*, 2011, **119**, 218–23.
8. B. Sadikovic and D. I. Rodenhiser, *Toxicol. Appl. Pharmacol.*, 2006, **216**, 458–68.
9. M. Toyota and H. Suzuki, *Adv. Genet.*, 2010, **70**, 309–23.
10. J. N. Filkowski, Y. Ilnytskyy, J. Tamminga, I. Koturbash, A. Golubov, T. Bagnyukova, I. P. Pogribny, and O. Kovalchuk, *Carcinogenesis*, 2010, **31**, 1110–5.
11. C. Yauk, A. Polyzos, A. Rowan-Carroll, C. M. Somers, R. W. Godschalk, F. J. Van Schooten, M. L. Berndt, I. P. Pogribny, I. Koturbash, A. Williams, G. R. Douglas, and O. Kovalchuk, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 605–10.
12. C. Vilariño-Güell, A. G. Smith, and Y. E. Dubrova, *Mutat. Res.*, 2003, **526**, 63–73.
13. Y. E. Dubrova, A. J. Jeffreys, and A. M. Malashenko, *Nat. Genet.*, 1993, **5**, 92–4.
14. C. L. Yauk, A. Polyzos, A. Rowan-Carroll, I. Kortubash, A. Williams, and O. Kovalchuk, *Environ. Mol. Mutagen.*, 2008, **49**, 26–35.
15. S. M. Mirkin, *Nature*, 2007, **447**, 932–40.
16. D. L. Cheo, H. J. Ruven, L. B. Meira, R. E. Hammer, D. K. Burns, N. J. Tappe, A. A. van Zeeland, L. H. Mullenders, and E. C. Friedberg, *Mutat. Res.*, 1997, **374**, 1–9.
17. R. W. Godschalk, I. T. Vermeer, E. Kriek, B. Floot, P. A. Schilderman, E. J. Moonen, J. C. Kleinjans, and F. J. van Schooten, *Chem. Biol. Interact.*, 1997, **104**, 41–54.
18. I. P. Pogribny, S. A. Ross, V. P. Tryndyak, M. Pogribna, L. A. Poirier, and T. V Karpinets, *Carcinogenesis*, 2006, **27**, 1180–6.
19. R. Kelly, G. Bulfield, A. Collick, M. Gibbs, and A. J. Jeffreys, *Genomics*, 1989, **5**, 844–56.
20. M. Gibbs, A. Collick, R. G. Kelly, and A. J. Jeffreys, *Genomics*, 1993, **17**, 121–8.
21. C. Paul and B. Robaire, *Nat. Rev. Urol.*, 2013, **10**, 227–34.
22. J. O. Linschooten, N. Verhofstad, K. Gutzkow, A.-K. Olsen, C. Yauk, Y. Oligschläger, G. Brunborg, F. J. van Schooten, and R. W. L. Godschalk, *FASEB J.*, 2013, **27**, 2873–9.
23. C. L. Yauk, M. L. Berndt, A. Williams, A. Rowan-Carroll, G. R. Douglas, and M. R. Stämpfli, *Cancer Res.*, 2007, **67**, 5103–6.
24. T. M. Singer, I. B. Lambert, A. Williams, G. R. Douglas, and C. L. Yauk, *Mutat. Res.*, 2006, **598**, 164–93.
25. L. Miccoli, K. L.-A. Burr, P. Hickenbotham, E. C. Friedberg, J. F. Angulo, and Y. E. Dubrova, *Cancer Res.*, 2007, **67**, 4695–9.
26. E.-S. A. Mohamed, W.-H. Song, S.-A. Oh, Y.-J. Park, Y.-A. You, S. Lee, J.-Y. Choi, Y.-J. Kim, I. Jo, and M.-G. Pang, *Hum. Reprod.*, 2010, **25**, 2427–33.
27. R. Tripathi, D. P. Mishra, and C. Shaha, *J. Reprod. Immunol.*, 2009, **83**, 31–5.
28. A. Revel, H. Raanani, E. Younglai, J. Xu, R. Han, J. F. Savouret, and R. F. Casper, *Reprod. Toxicol.*, **15**, 479–86.
29. G. Barreto, A. Schäfer, J. Marhold, D. Stach, S. K. Swaminathan, V. Handa, G. Döderlein, N. Maltry, W. Wu, F. Lyko, and C. Niehrs, *Nature*, 2007, **445**, 671–5.
30. J. P. M. Melis, S. W. P. Wijnhoven, R. B. Beems, M. Roodbergen, J. van den Berg, H. Moon, E. Friedberg, G. T. J. van der Horst, J. H. J. Hoeijmakers, J. Vijg, and H. van Steeg, *Cancer Res.*, 2008, **68**, 1347–53.
31. J. P. M. Melis, M. Luijten, L. H. F. Mullenders, and H. van Steeg, *Mutat. Res.*, **728**, 107–17.
32. J. del Mazo, G. Prantera, M. Torres, and M. Ferraro, *Chromosome Res.*, 1994, **2**, 147–52.
33. V. L. Wilson and P. A. Jones, *Cell*, 1983, **32**, 239–46.
34. J. Bao and W. Yan, *Biol. Reprod.*, 2012, **86**, 162, 1–14.
35. V. Stribinskis and K. S. Ramos, *Cancer Res.*, 2006, **66**, 2616–20.
36. D. C. Hancks and H. H. Kazazian, *Curr. Opin. Genet. Dev.*, 2012, **22**, 191–203.

37. H. Kano, I. Godoy, C. Courtney, M. R. Vetter, G. L. Gerton, E. M. Ostertag, and H. H. Kazazian, *Genes Dev.*, 2009, **23**, 1303–12.
38. G. Ji, L. Yan, W. Liu, J. Qu, and A. Gu, *Toxicol. Lett.*, 2013, **218**, 144–9.
39. J. O. Linschooten, J. Laubenthal, E. Cemeli, A. Baumgartner, D. Anderson, V. E. Sipinen, G. Brunborg, G. R. M. M. Haenen, E. Fthenou, J. J. Briedé, F. J. van Schooten, and R. W. L. Godschalk, *Reprod. Toxicol.*, 2011, **32**, 106–11.
40. S. Horak, J. Polanska, and P. Widlak, *Mutat. Res.*, 2003, **537**, 53–65.
41. W. A. Robbins, D. A. Elashoff, L. Xun, J. Jia, N. Li, G. Wu, and F. Wei, *Cytogenet. Genome Res.*, 2005, **111**, 371–7.

Table 1A. Methylation of LINE1-ORF2, SINEB1 and SINEB2 in the testes of B[a]P exposed fathers with different *Xpc*-genotypes and their wildtype counterparts (Average± SE), related to unexposed animals of the same genotype (reference). 1B. Methylation of LINE1-ORF2, SINEB1 and SINEB2 in liver of offspring animals from B[a]P exposed fathers.

A.

	LINE1	SINE B1	SINE B2	Combined
Testis Father	**Relative DNA methylation**	**Relative DNA methylation**	**Relative DNA methylation**	**Relative DNA methylation**
Wt control	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Wt exposed	0.12 ± 0.05 *	0.65 ± 0.14	0.55 ± 0.07 *	0.43 ± 0.16 *
***Xpc*$^{-/-}$ control**	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
***Xpc*$^{-/-}$ exposed**	0.49 ± 0.14	0.59 ± 0.06	0.95 ± 0.05	0.64 ± 0.08 *

* P<0.05 compared to unexposed controls carrying the same genotype.

B.

	LINE1	SINE B1	SINE B2
Liver Offspring	**Relative DNA methylation**	**Relative DNA methylation**	**Relative DNA methylation**
Wt control	1.00 (ref)	1.00 (ref)	1.00 (ref)
Wt exposed	0.86 ± 0.09	1.47 ± 0.15	0.91 ± 0.24
***Xpc*$^{-/-}$ control**	1.00 (ref)	1.00 (ref)	1.00 (ref)
***Xpc*$^{-/-}$ exposed**	0.74 ± 0.16	1.17 ± 0.22	1.13 ± 0.13

Table 2. ESTR mutation rates in offspring of B[a]P exposed and control male mice.

Group	Pups scored	Paternal Mutations*	Paternal Mutation rate	Ratio to control (CI)	Fisher's exact test P value
Wt B[a]P	85	5	0.029 ± 0.018	1.05 (0.03, 3.05)	0.50
Wt Control	89	5	0.028 ± 0.017		
***Xpc*$^{-/-}$ B[a]P**	134	15	0.056 ± 0.020	3.25 (0.25, 9.95)	0.034
***Xpc*$^{-/-}$ Control**	87	3	0.017 ± 0.013		

* Singleton mutations only: data are combined for single loci *Ms6-hm* and *Hm-2*.

Fig. 1. DNA fingerprint of a mouse family exposed to B[a]P for ESTR locus *Ms6-hm*. Parents are labeled ♂ (father) and ♀ (mother) and pups are labeled 1 to 7. Pup 7 has a mutation in the paternal allele (as indicated by *) because the allele size is different from the two alleles found in the father. Two mothers are indicated on the gel, because each father was mated with two females. Size range is indicated with a 1kb ladder (kb).