Age, sex and co-exposure to N-ethyl-N-nitrosourea influence mutations in the Alu repeat sequences in diethylstilbestrol-induced kidney tumors in Syrian hamsters

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We report, for the first time, mutations in the Alu repeat regions in the genome of kidney tumors induced by diethylstilbestrol in Syrian hamsters. Among the 66 loci amplified by 11 random primers, 28 loci exhibited insertions, deletions or losses or gains in intensity in the genome of kidney tumor tissues compared with normal kidney tissues from age-matched hamsters. Higher numbers of mutated Alu loci were observed in the tumors of old hamsters compared with young hamsters. In N-ethyl-N-nitrosourea- and diethylstilbestrol-treated hamsters deletion of a 0.59 kb locus amplified with primer OPC03 was observed in most of the female hamsters, but not in male hamsters. An insertion mutation of a 0.498 kb locus amplified with primer OPC03 was observed in 12 of 36 diethylstilbestrol-induced kidney tumors. The cloning and sequencing of the 0.498 kb locus amplified with primer OPC03 revealed that it had significant sequence similarity to the mouse RIKEN cDNA clone. These findings indicate that age, sex and co-exposure to N-ethyl-N-nitrosourea influence mutations in the Alu repeat sequences in the genome of diethylstilbestrol-induced kidney tumors in Syrian hamsters. Structural alterations in Alu repeats in critical target genes may be involved in diethylstilbestrol-induced carcinogenesis.

Introduction

Ongoing epidemiological studies suggest a slightly increased threat of breast cancer in women exposed to synthetic diethylstilbestrol (DES) (Laitman, 2002). There is also growing concern that DES may be involved in the development of some disorders of the male reproductive system, including increased occurrence of testicular cancers (Laitman, 2002). The treatment of male Syrian hamsters with natural estrogen, 17β-estradiol (E2) or DES produces a 90–100% incidence of kidney tumors (Kirkman, 1972; Li and Nandi, 1990; Roy and Liehr, 1990; Narayan and Roy, 1993; Llombart-Bosch et al., 1996; Roy et al., 1996). The mechanism by which synthetic or natural estrogen causes cancer is not clear. Steroidal estrogen-induced genotoxicity in Syrian hamster embryonic, uterine and breast cells and hpmt mutations in Chinese hamster V79 cells have recently been reported (Kong et al., 2000; Tsutsui et al., 2000; Liehr, 2001). DNA repair activity is altered by exposure to DES (Yan and Roy, 1995; DuMond and Roy, 2001). Kidney tumors and estrogen-treated kidneys have mutations in microsatellites (Hodgson et al., 1998). Despite extensive research in this experimental model of hormonal carcinogenesis, target candidate regions of the genome susceptible to mutations in response to exposure to DES or E2 are yet to be identified.

Mutations in the repeat sequence regions in the genome of both human and animal tumors have been very frequently observed. Inter-Alu PCR has been used to identify gene loci sensitive to replication errors in hereditary non-polyposis colon cancer and to detect genetic alterations in B cell lymphoma (Krajnovic et al., 1996; Bertoni et al., 2000). Inactivation of the P53 tumor suppressor gene and up-regulation of the ART1 gene due to a rearrangement involving Alu elements in human pancreatic cancer cells have been identified by Alu-PCR fingerprinting (McKie et al., 1997; Slebos et al., 1998). These reports of a high incidence of mutations in Alu repeat sequences in various cancers and the highly ubiquitous distribution of Alu repeats in the genome indicate that targeting this region would have a greater probability of detecting mutations in target candidate regions in the genome or the weakly mutagenic activity of carcinogenic estrogenic chemicals, such as DES.

We have recently optimized and successfully used random amplified polymorphic DNA (RAPD)/AP-PCR fingerprinting for the identification of mutations in human as well as estrogen-induced cancers in an experimental model (Singh and Roy, 1999). Using this method, we have identified mutations in a novel uncharacterized gene in human sporadic breast cancer (Singh and Roy, 2001). The incidence of genomic instability in ovarian cancer has been found to be much higher with Alu/PCR than with traditional PCR-amplified microsatellite markers (Sood and Buller, 1996). Therefore, in this study we have used Alu/RAPD-PCR fingerprinting to detect mutations in the genome of DES-induced kidney tumors. We report for the first time the influence of age, sex and co-exposure to N-ethyl-N-nitrosourea (ENU) on Alu repeat sequences in the genome of DES-induced hamster kidney tumors. Although, the exact functional importance of mutated Alu repeat sequence-containing loci is unknown, this study indicates that these altered Alu loci may participate in the progression step of DES-induced carcinogenesis.

Materials and methods

Chemicals and reagents

Sets of 20 OPA, OPC, OPK, OPE, OPAA and OP26 random 10mer oligonucleotide primers were purchased from Operon Technologies (Alameda, CA). AmpliTaq DNA polymerase (recombinant), dNTPs and mineral oil were purchased from Applied Biosystems (Foster City, CA). The enzyme assay buffers, 10× PCR buffer II (500 mM KCl, 100 mM Tris–HCl, pH 8.3) for AmpliTaq and 10× Stoffel buffer (100 mM KCl, 100 mM Tris–HCl, pH 8.3) were received from Applied Biosystems along with the corresponding enzymes.

DNA extraction

Male Syrian hamsters (6–8 weeks old) received s.c. implants of a 25 mg DES pellet every 2 months (Narayan and Roy, 1993; Llombart-Bosch et al., 1996;

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The s.c. implants maintain a steady-state serum estrogen level of 2400±2700 pg/ml (Li et al., 1994). The control group of animals (n = 6) received cholesterol pellets. Nineteen animals were also treated with DES in combination with ENU (Peydro-Olaya et al., 2001). Numbers of animals in each group and dose and duration of treatment are described in Table I. Genomic DNA was extracted from age-matched untreated controls and tumor tissues by the method described previously by us (Singh and Roy, 1999). The concentration of DNA was determined by spectrophotometry. The quality of DNA was checked by ethidium bromide staining after resolution on a 1% agarose gel.

RAPD-PCR

RAPD-PCR was performed on DNA from untreated control kidney tissue and DES-induced tumors using a previously described method (Singh and Roy, 1999). An aliquot of 2 μg DNA was incubated separately with the restriction enzymes AluI, EcoRI, BamHI, HindIII and HinfI at 37°C. After overnight incubation for complete digestion of DNA, the restriction enzyme was heat-denatured by incubation at 70°C for 10 min. The digested DNA samples were then diluted to 20 ng/μl for RAPD analysis. Briefly, the PCR amplifications were performed in 25 μl of reaction mixture containing 2.5 μl of 10% enzyme assay buffer, 100 μM each of dATP, dCTP, dGTP and dTTP, 100 nM random (10 bp) primer, 1.5 mM MgCl₂, 1.5 U AmpliTaq DNA polymerase or Stoffel fragment and 75 ng either undigested or restriction enzyme-digested DNA as template. The amplification was performed in a Perkin-Elmer Cetus DNA thermal cycler programmed for 45 cycles as follows: one cycle of 3.5 min at 92°C, 1 min at 34°C, 2 min at 72°C, followed by 44 cycles of 1 min at 92°C, 1 min at 34°C, 2 min at 72°C, with a final extension of 15 min at 72°C. Amplified products were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

Cloning and sequencing of RAPD loci

Amplified fragment was excised from the agarose gel and DNA was eluted. The eluted DNA was reamplified with the same random primer and using the same concentrations of reaction mixture constituents and PCR cycle conditions as described above. The PCR products were analyzed on an agarose gel to confirm their size and purity. The reamplified DNA fragments were cloned using a TA cloning kit (Invitrogen, Carlsbad, CA), following the protocol provided by the manufacturer. Restriction analysis of the recombinant plasmid DNA was performed by the alkaline lysis method to confirm the insert size. The presence of the correct insert was further confirmed by hybridization with the RAPD-eluted DNA fragment. The cloned RAPD-PCR product was sequenced by the DNA Sequencing Core Facility (Comprehensive Cancer Center, University of Alabama at Birmingham). Sequences obtained from our clones were compared with known sequences in the GenBank database using the BLASTn and BLASTx programs.

Table I. Dose and duration of treatment of Syrian hamsters

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of animals</th>
<th>Dose (mg)</th>
<th>Age/treatment (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (8, 5 males and 3 females)</td>
<td>M (9)</td>
<td>210–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M (2)</td>
<td>498–553</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F (3)</td>
<td>553–564</td>
<td></td>
</tr>
<tr>
<td>DES (17, 11 males and 6 females)</td>
<td>M (2)</td>
<td>210/180</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M (3)</td>
<td>248–316/93–226</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M (4)</td>
<td>364–403/216–290</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M (2)</td>
<td>417–432/284–304</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F (6)</td>
<td>307–334/213–240</td>
<td></td>
</tr>
<tr>
<td>DES + ENU (19, 12 males and 7 females)</td>
<td>F (1)</td>
<td>111/11/16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F (2)</td>
<td>253–386/166–299 + 180–313</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F (4)</td>
<td>258–336/187–265 + 201–279</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M (1)</td>
<td>346/259 + 273</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M (6)</td>
<td>373–420/286–316 + 300–330</td>
<td></td>
</tr>
</tbody>
</table>

M, male; F, female; DES, diethylstilbestrol; ENU, N-ethyl-N-nitrosourea.

Table II. Summary of the insertion, deletion and intensity differences in the AluI repeat sequences containing loci from diethylstilbestrol-induced kidney tumors compared with age-matched controls

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Total amplification products (variable)</th>
<th>Size (kb) of variable amplification products in tumor DNA</th>
<th>Deletion</th>
<th>Insertion</th>
<th>Intensity difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Y</td>
<td>O</td>
<td>Y&amp;O</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Y</td>
<td>O</td>
<td>Y&amp;O</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Y</td>
<td>O</td>
<td>Y&amp;O</td>
</tr>
<tr>
<td>OPA05</td>
<td>AGGGGTCTTG</td>
<td>5 (2)</td>
<td>0.51</td>
<td></td>
<td>0.60</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>OPA10</td>
<td>GTGATCCGAG</td>
<td>8 (6)</td>
<td>0.80</td>
<td>0.60</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>OPA15</td>
<td>TTTCCGAACCC</td>
<td>6 (2)</td>
<td>0.31</td>
<td>0.59</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>OPA16</td>
<td>AGCCAGCGAA</td>
<td>8 (3)</td>
<td>0.62</td>
<td>0.59</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>OPC03</td>
<td>GGGGGTCTTT</td>
<td>3 (2)</td>
<td>0.59</td>
<td>0.59</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>OPE11</td>
<td>GAGTCTCAGG</td>
<td>4 (1)</td>
<td>0.60</td>
<td>0.59</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>OPK03</td>
<td>CCAGCTTGG1</td>
<td>3 (1)</td>
<td>0.61</td>
<td>0.60</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>OPA001</td>
<td>AGACGGCTCC</td>
<td>11 (4)</td>
<td>0.61</td>
<td>0.60</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>OPA007</td>
<td>CTACGCTCAC</td>
<td>4 (2)</td>
<td>0.49</td>
<td>0.50</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>OPA009</td>
<td>AGATGGGCGCAG</td>
<td>8 (1)</td>
<td></td>
<td>0.50</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>OPE26-1</td>
<td>TACAACGAG</td>
<td>6 (4)</td>
<td>0.19</td>
<td>0.61</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Sizes of the amplification products are given in kilobases. Y, loci from young animal; O, loci from old animal; Y&O, loci common in young and old animals.

*aLoss in intensity.
*bGain in intensity.
Results

Tumor-specific changes in RAPD loci

Initially, 15 random 10mer primers were used for Alu-RAPD PCR fingerprinting. Of these, 12 produced reproducible and scorable amplification products. One of the 12 primers (OPC11) produced similar RAPD fingerprints for controls and tumors, rendering it uninformative in revealing alterations in tumor DNA. The remaining 11 primers detected changes in the RAPD profiles of the tumors compared with untreated controls. The sequence of these 11 primers, total number of amplification products generated by these individual primers and variable fragments in DES and/or DES and ENU co-treated hamster kidney tumors and untreated controls are described in Table II and representative RAPD fingerprints are shown in Figures 1±4. A total of 66 amplification products (loci) were observed by the 11 primers, with an average of 6.0 loci/primer. The total number of loci amplified by an individual primer ranged from 3 in OPK03 to 11 in OPAA01 (Figure 1 and Table II). Of the 66 loci that were amplified, 28 (42.42%) were variable between kidney tumors induced by DES alone or DES plus ENU and untreated controls. Most importantly, primer C03 revealed insertion of a 0.498 kb locus in 12 of 36 tumor samples as compared with controls (Figure 3). Irrespective of the age and sex of the animals, as well as the dose, duration and type of treatment (DES alone or DES in combination of ENU), the 0.498 kb locus was inserted in 12 of 36 tumor samples.

Age-specific differences in gene loci in tumors

Tumors from animals of two different ages (210 and 432 days) exposed for the same duration to DES showed variable amplification products (Table II). These age-specific tumor-associated loci were of two types.

Mutated loci in tumors from old animals. Comparison of RAPD fingerprints of pooled tumors from three old animals (432 days) with those from three young animals (210 days) and controls revealed insertions of 0.70 and 0.61 kb loci amplified with primer OPA10, a 0.41 kb locus amplified with primer OPA16 and 0.80 and 0.59 kb loci amplified with primer OPE26-1. Deletions of 0.61 and 0.80 kb loci amplified with primers OPAA01 and OPA10, respectively, were found only in tumors from old animals, but not in tumors from young animals or controls (Figures 1 and 2). Similarly, a reduction in the intensity of a 0.42 kb locus amplified with primer OPK03 and a gain in intensity of a 0.40 kb locus amplified with primer OPAA01 were found only in tumors from old animals and not in tumors from young animals and controls (Figure 1). Since the DES exposure period was similar in both young and old animals bearing tumors, higher mutated loci in tumors from old animals may be the result of the combined influences of senescence and DES.

Young age-specific loci in tumors. Insertions of 0.60 and 0.31 kb loci amplified with primer OPA10 and a 0.59 kb locus amplified with primer OPA16 were observed only in RAPD fingerprints of tumors from young age animals as compared with either controls or tumors from old animals (Figures 1 and 2). Absence of these amplified loci harboring mutations in tumors from old animals indicates that DES treatment of young and old animals produces different types of mutations.

Sex-specific differences in gene loci in DES-ENU-induced tumors

Comparison of the RAPD fingerprints generated by primer OPC03 from kidney DNA of 19 animals (5 controls and 14 tumors) revealed that a 0.59 kb locus was present in kidney DNA from both male and female untreated hamsters (Figure 3, lanes 5±9). This amplified locus was absent in five out of six DNA samples from ENU + DES-induced kidney tumors in female hamsters, whereas this RAPD locus was present in kidney DNA of male hamsters treated with ENU + DES (Figure 3, lanes 19, 21 and 22). However, 80% of the male

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Fig. 1. Representative RAPD fingerprints from DNA of DES-induced kidney tumors pooled from three 432-day-old male hamsters (T₀) and from three 210-day-old male hamsters (T₁) and age-matched controls (C). OPA10, OPAA01 and OPK03 represent primers used to generate the fingerprints.
hamsters treated with DES alone (four of five) showed deletion of this locus (Figure 3, lanes 10, 12, 13 and 16). Fisher’s exact test showed a significant difference in absence of the 0.59 kb PCR amplified locus between the female and male groups of ENU + DES-treated animals (P < 0.05). Thus, it appears that a 0.59 kb locus amplified by primer OPC03 is more susceptible to mutations in females co-treated with nitrosourea than male hamsters.

Cloning, sequencing and sequence homology search of the tumor-specific 498 bp RAPD locus amplified by the OPC03 primer

The tumor-specific 498 bp RAPD locus amplified with primer OPC03 was further cloned and sequenced (Figure 5). Sequence homology searches revealed that the sequence of the 498 bp amplified fragment had significant similarity (84% identity) with the RIKEN cDNA clone, accession no. BB612168.

Discussion

This is the first report of mutational analysis of Alu repeat sequences in DES-induced Syrian hamster kidney tumors. The important finding that emerged from this study is that DES-induced tumors showed both qualitative (insertion/deletion, presumably as a result of point mutations or length mutations) and quantitative changes (intensity changes, presumably as a result of aneuploidy or polyploidy) in Alu-PCR fingerprints. The mutations detected were of two types, one that was very frequent among the DES-induced tumors and another that was present in a few selected tumor samples. The mutation that was common in many tumors indicates that it is associated with DES-induced tumorigenesis. Those mutations that were uniquely present in some tumors were associated with either the age or the sex of the animals. Deletion of a 590 bp locus amplified with primer OPC03 observed mostly in female rather
than male hamsters indicates that this Alu repeat region is presumably more susceptible to DES-induced mutations in the female than in the male genome. Although the exact functional importance of mutated Alu-containing loci is unknown, these altered Alu loci may provide suggestive evidence for loci that participate in tumorigenesis. These mutations were detected only with restriction enzyme AluI digested DNA as template in the RAPD. These loci presumably harbored mutations in the AluI restriction enzyme recognition sequence. Thus, our approach of combining RFLP with RAPD has an advantage over classical RAPD fingerprinting in detecting mutations. Thus Alu/RAPD-PCR may be useful in searching for alterations in archived samples from women with DES-linked cancers and in those individuals who are at high risk of developing DES exposure-associated cancer.

Products of the redox cycling of DES generate free radical-driven DNA adducts as well as DES–DNA adducts (Liehr and Roy, 1990; Liehr and Roy, 1998; Cavaleri et al., 2000). An increase in the 8-hydroxyguanine base content of kidney DNA has been observed after chronic treatment of hamsters with estrogen (Roy et al., 1991). The DES quinone reactive metabolite increases homologous recombination in Escherichia coli (Korah et al., 1993). Both DES and E2 are mutagenic in the gpt+ Chinese hamster cell line G12 (Klein, 1995). Covalent DNA adducts, formed by both the DES quinone and E2 quinone, arrest the progression of DNA synthesis (Roy and Abul-Hajj, 1997). Thus, it is possible that the covalent interaction of DNA bases with active DES metabolites or reactive free radical species generated during DES redox reactions are responsible for the observed mutations in the Alu repeat sequences. However, that these mutations could also be due to genomic instability caused by tumor progression, rather than directly due to DES-induced genotoxicity, cannot be ruled out.

Alu repeat sequences/elements were initially discovered by the digestion of human DNA with the restriction enzyme AluI (Slebos et al., 1998). The Alu repeat is one of the most common repeat elements in the human as well as the hamster genome (Nelson et al., 1989), and these sequences are hotspots for genomic instability (Mighell et al., 1997). Gene sequence rearrangements through recombination involving Alu elements have been implicated in the inactivation of the P53 tumor suppressor gene (Slebos et al., 1998) and low density lipoprotein receptor gene (Lehrman et al., 1985). Alu elements have also been implicated in the production of oncogenes as a result of chromosomal translocation events joining the BCR and ABL loci and the LCK and TCRB loci (Tycko et al., 1991; Chissoe et al., 1995). Alu-associated recombination occurring
in the germline has also been reported in human familial cancer (Marshall et al., 1996). Cloning and sequencing the amplified OPC03 0.498 kb locus detected in this study revealed that this mutation is in a coding region of the genome. The sequence of the 498 bp Alu/RAPD product amplified by the OPC03 primer had significant homology with the mouse RIKEN cDNA clone and amplification of this sequence could have functional significance in DES-induced carcinogenesis. Our present findings indicate that novel Alu repeat-containing genes susceptible to mutation during DES-induced tumorigenesis can be identified using this Alu/RAPD screening method. Further characterization of this and additional target sites may lead to the identification of genes susceptible to mutation and provide clues as to the mechanism of DES-induced tumorigenesis.

Other novel findings that emerged from this study is a deletion mutation of the OPC03 0.59 kb Alu-containing locus as a result of ENU + DES treatment, which was present in 83% of the female hamsters but not in male hamsters, and that a higher number of mutated Alu loci occurred in tumors from old hamsters than in tumors from young hamsters. It has recently been reported that sex differences influence the rate of mutation in repeat sequences (IHGSC, 2001). It is well known that aging significantly increases the risk of cancer in both humans and rodents (DePinho, 2000; Uys and Van Helden, 2003). Somatic mutations are frequent and increase with age in human kidney epithelial cells (Martin et al., 1996). The incidence of kidney tumors is higher in female rats exposed to ENU and E2 than male rats (Aoyama et al., 1989). From the findings of this study it appears that sex differences affect the rate of mutation in Alu repeat sequences induced by chemicals such as DES and ENU. The significantly higher incidences of ENU-induced kidney tumors in female than male rodents observed previously may be the result of higher rates of mutation in ENU-treated females than males. Our present findings of higher incidences of mutations in tumors from older animals and the previous reports of higher incidences of cancers in older animals clearly indicate that age also influences the rate of chemical-induced mutations and cancers. Whether these tumors associated with old age and sex are involved in the causation of DES-induced carcinogenesis or merely represent tumor cell instability remains to be ascertained.

In summary, we report for the first time that age, sex and co-exposure to N-ethyl-N-nitrosourea influence mutations in the Alu repeat sequences in the genome of DES-induced hamster kidney tumors. Structural alterations in Alu repeats in target genes may be involved in DES-induced carcinogenesis. Further identification and characterization of the genes as well as regulatory parts of the susceptible regions of Alu-containing genes affected by DES exposure are currently in progress to fully understand DES-induced carcinogenesis.

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