Research

Somatic mutations in stilbene estrogen-induced Syrian hamster kidney tumors identified by DNA fingerprinting Kamaleshwar P Singh and Deodutta Roy*

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Abstract

Kidney tumors from stilbene estrogen (diethylstilbestrol)-treated Syrian hamsters were screened for somatic genetic alterations by Random Amplified Polymorphic DNA-polymerase chain-reaction (RAPD-PCR) fingerprinting. Fingerprints from tumor tissue were generated by single arbitrary primers and compared with fingerprints for normal tissue from the same animal, as well as normal and tumor tissues from different animals. Sixty one of the arbitrary primers amplified 365 loci that contain approximately 476 kbp of the hamster genome. Among these amplified DNA fragments, 44 loci exhibited either qualitative or quantitative differences between the tumor tissues and normal kidney tissues. RAPD-PCR loci showing decreased and increased intensities in tumor tissue DNA relative to control DNA indicate that loci have undergone allelic losses and gains, respectively, in the stilbene estrogen-induced tumor cell genome. The presence or absence of the amplified DNA fragments indicate homozygous insertions or deletions in the kidney tumor DNA compared to the age-matched normal kidney tissue DNA. Seven of 44 mutated loci also were present in the kidney tissues adjacent to tumors (free of macroscopic tumors). The presence of mutated loci in uninvolved (non-tumor) surrounding tissue adjacent to tumors from stilbene estrogen-treated hamsters suggests that these mutations occurred in the early stages of carcinogenesis. The cloning and sequencing of RAPD amplified loci revealed that one mutated locus had significant sequence similarity with the hamster Cyp |A| gene. The results show the ability of RAPD-PCR to detect and isolate, in a single step, DNA sequences representing genetic alterations in stilbene estrogeninduced cancer cells, including losses of heterozygosity, and homozygous deletion and insertion mutations. RAPD-PCR provides an alternative molecular approach for studying cancer cytogenetics in stilbene estrogen-induced tumors in humans and experimental models. Although the exact functional importance of mutated loci is unknown, this study indicates that these altered loci may participate during tumor progression in the kidney.

Introduction

Estrogens are carcinogenic to both humans and experimental animals [1,2]. This is due to their ability to initiate carcinogenesis and promote the growth and selection that result in palpable malignancy [2,3]. For example, the treatment of male Syrian hamsters with natural estrogen (17 ß estradiol, E_2) or synthetic stilbene estrogen (diethyl-stilbestrol, DES) produces a 90–100% incidence of kidney

tumors [3,4]. Ongoing epidemiological studies suggest a slightly increased threat of breast cancer in women exposed to DES [1,2]. There is also a growing concern that DES may be involved in the development of some disorders of the male reproductive system, including an increased occurrence of testicular cancers [2]. Both E_2 and DES induce cell transformation [5,6]. However, the mechanisms by which stilbene estrogen or natural estrogen cause cell transformation and progression of transformed cells are not clear.

Treatment of hamsters with DES produces both numerical (polyploidy, aneuploidy) and structural (deletions, inversions, translocations) changes in the genome of renal cells [7,8]. Aneuploidy has been detected in SHE cells transformed by both E₂ and DES [9,10]. In addition to chromosomal aberrations, biochemical changes in the hamster kidney suggest that DES treatment produces changes at the genome level [11-13]. Both DES and its metabolites alter transcriptional activity [14]. Attenuation in DNA repair coupled with an increase in renal tubular cell proliferation in response to DES exposure may lead to enhanced genetic instability. DES and/or DES metabolites may interfere with the spindle apparatus during mitosis resulting in abnormal segregation of chromosomes [15]. Products of the redox cycling of DES generate free-radicaldriven DNA adducts as well as DES-DNA adducts [16,17]. Chronic treatment of hamsters with DES results in an increase in the 8-hydroxyguanine in kidney DNA [18]. Despite extensive work on DES, the critical sequence of molecular events involved in DES-induced carcinogenesis is not clear.

All human tumors contain genetic alterations. Mutations in a minimum of six to seven genes are crucial to the process of cancer development [19]. Detection of the genomic regions that are susceptible to stilbene estrogen-induced mutation is a critical step for the identification of the genes that may play a role in stilbene estrogen-induced carcinogenesis. Steroidal estrogen produces genotoxicity in Syrian hamster embryonic cells, uterine and breast cells, and induces Hprt mutations in Chinese hamster V79 cells [20-22]. Recent studies show that the reactive DES metabolite, quinone, increases homologous recombination in E. coli [23]; both DES and E2 are mutagenic in the gpt+ Chinese hamster G12 cell line [24]; covalent DNA adducts formed by both DES quinone and E2 quinone arrest the progression of DNA synthesis [25]; DNA repair activity is altered by exposure to DES [26,27]; and kidney tumors and estrogen-treated kidneys have mutations in microsatellites [28].

We recently have optimized the Random Amplified Polymorphic DNA (RAPD) reaction conditions for both human and experimental animal DNA [29]. RAPD-PCR

can detect allele losses and gains as well as deletions and insertions of DNA sequences; thus its resolution is at the molecular level. Using this method, we have identified mutations in a novel uncharacterized gene in human sporadic breast cancer [30]. In this study, we have used RAPD-PCR to identify candidate target regions for mutations in the genome of hamster kidney tumors induced by stilbene estrogen. We screened 365 loci in DNA from Syrian hamster kidney tumors, observing insertion, deletion, or intensity differences in 44 loci. We also examined alterations in the genome of uninvolved (non-tumor) surrounding kidney tissue adjacent to tumors with the objective of screening for molecular lesions that occurred before the appearance of tumors. Finding common mutated loci in tumors and uninvolved (non-tumor) surrounding kidney tissue adjacent to tumors indicates that these mutations may be involved in the early steps of stilbene estrogen-induced carcinogenesis.

Materials and methods Chemicals and Reagents

Sets of twenty OPA, OPC, OPK, OPE, OPAA and OP 26 oligonucleotide random 10-mer primers were purchased from Operon Technologies (Alameda, CA). AmpliTaq DNA polymerase (recombinant), Stoffel fragment DNA polymerase, dNTPs and mineral oil were purchased from Applied Biosystems (Foster City, CA). The enzyme assay buffers, 10X PCR buffer II (500 mM KCl, 100 mM Tris-HCl, pH 8.3) for AmpliTaq and 10X Stoffel buffer (100 mM KCl; 100 mM Tris-HCl, pH 8.3), were received from Applied Biosystems along with the corresponding enzymes.

Tumor induction and DNA extraction

Male Syrian hamsters (6-8-weeks old) received a subcutaneous implant of a 22-mg DES pellet every three months [3]. The subcutaneous implant maintains a steady-state serum estrogen level of 2400 to 2700 pg/ml [4]. After 7 months of treatment, 10 animals were sacrificed, and their kidneys were excised. Tumors and uninvolved surrounding tissues free of visible tumors from the kidneys were dissected out and frozen in liquid nitrogen. Since the tumors are small after 7 months of treatment, we pooled tumors and uninvolved surrounding tissues from the same treated animals for DNA isolation. We used three groups of tumor DNA, and each group contained DNAs from several animals. The control group of six animals received cholesterol pellets. We separated control tissues into three groups; each group containing pooled tissues from two control animals. Nuclear DNA was extracted from age-matched untreated control, DES-induced tumor tissues, and uninvolved (non-tumor) surrounding tissue adjacent to tumors. 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 [29]. An aliquot of 2 µg DNA was incubated separately with the restriction enzymes Alu I, 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 10X enzyme assay buffer, 100 µM each of dATP, dCTP, dGTP and dTTP, 100 nM of random (10-bp) primer, 1.5 mM MgCl₂, 1.5 U of AmpliTaq DNA polymerase and 75 ng of either undigested or restriction enzymedigested DNA as template. We used Stoffel fragment instead of AmpliTaq in Alu digested DNA template. The amplification was performed in a Perkin-Elmer Cetus DNA thermal cycler programmed for 45 cycles as follows: 1st cycle of 3.5 min at 92°C, 1 min at 34°C, 2 min at 72°C, plus 44 cycles of 1 min at 92°C, 1 min at 34°C, 2 min at 72°C, followed by a final extension cycle 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 fragments were 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 cycles 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 the 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 [31]. 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, UAB. Sequences obtained from our clones were compared with known sequences in the GeneBank database using the BLASTn and BLASTx programs.

Data analysis

The size of the genome screened was calculated by adding the sizes of the all the individual loci amplified by the random primers (365 loci amplified by 61 primers). The percent of the genome screened was calculated by dividing the size of the genome screened by the full length of the rodent genome \times 100. The size of the haploid hamster genome is estimated to be a similar to that of human, i.e., approximately 3×10^9 bp of DNA [32]. Each amplification product contains two 10-bp priming sites. Thus, we calculated the size of the genome screened for point mutations at the primer binding sites as the number of amplification products $\times (10 + 10)$ bp. The size of the genome screened for length mutation is the value obtained by deducting the size of the priming sites from total size screened. Loss of product intensity is associated with copy-number change as a result of hypoploidy [33]. Similarly gain of product intensity is associated with copy-number change due to hyperploidy. The percentage of the genome harboring point mutation, length mutation, hypoploidy and hyperploidy was calculated by considering the total length screened (475,630 bp) as the base value.

Results

Seventy random 10-mer primers were used to analyze instability in the genome of kidney tumors using RAPD-PCR fingerprinting. These initial screens were conducted using DNAs that were not digested with restriction endonucleases. Of the 70 primers, 61 produced reproducible and scorable amplification fingerprints. A total of 365 loci were amplified by the 61 primers, with an average of 6 loci per primer. The total length of the genome screened was approximately 476 kbp (Table 1). Of the 365 loci analyzed, 44 harbored mutations. The size of the 44 mutated loci was 50,568 bp, which is 10.63 % of the total length of the genome screened. The priming sites of the 365 amplified loci contain 7,300 bp (1.53%) of amplified sequence; the remaining DNA between the priming sites of the 365 loci contain 468,330 bp (98.47%). Of the 44 mutated loci, 18 were losses, 18 were gains, and the remaining 8 had intensity differences. In terms of size, the 18 lost loci contain 21,690 bp (4.56%), the 18 gained loci contain 21,158 bp (4.45%), the 1 hyperploid locus contains 700 bp (0.15%) and the 7 hypoploid loci contain 7,020 bp (1.48%)

Loci without mutation (i.e. similar in control and tumor DNA)

Representative photographs of monomorphic (similar loci) RAPD-PCR fingerprints for DES-induced tumors and age-matched controls are shown in Fig. 1. Out of the 61 primers, 42 (68.85%) amplified 266 loci that were similar in control and tumor DNA. The remaining 19 primers amplified a total of 99 loci, of which 55 were also similar in DNA from DES-induced tumors and age-matched controls. Although these loci were not informative in revealing alterations in tumor DNA, loci without mutations indicate that RAPD-PCR can detect not only mutated regions but also genomic regions that have not been affected by tumorigenesis. Thus, the simultaneous amplification of both the affected/susceptible region and the

Table 1: Summary of the changes in the genome of stilbene estrogen-induced kidney tumors compared to kidneys of age-match cont	rol
hamsters.	

Parameter Examined	Control	Tumor
Total number of loci screened	365	365
Number of loci with mutations	-	44
Length of the genome screened (in bp)	~475,630	~475,630 (0.016%)
Number of loci showing gains in intensity (hyperploidy)	-	l i i
Number of loci showing reductions in intensity (hypoploidy)	-	7
Number of loci gained (insertion)	-	18
Number of loci lost (deletion)	-	18



Figure I

Representative RAPD profiles showing similar DNA fingerprint patterns with DNA from stilbene estrogen-induced kidney tumors (T) and age-matched controls (C). Primers used (OPA 04, OPA 10, OPC 15, OPE 11, and OPK 03) are given at the bottom of each set of fingerprints.

unaffected region of the genome validates the sensitivity and reliability of RAPD-PCR fingerprinting.

Loci with mutations common to DES-induced tumors and surrounding uninvolved tissue adjacent to tumors

We observed 7 mutated loci amplified by 5 individual primers (OPA11, 18, 19; OPC14, OPE12) in tumor-free tissues that were adjacent to tumors, and these same loci were also altered in the adjacent DES-induced tumors. The sizes of these seven loci are indicated by bold-face type in Table 2, and the RAPD fingerprints are shown in Fig. 2. The mutations consisted of three losses (1.5- and 0.75-kbp fragments with primer OPA11 and a 0.6-kbp frag-

ment with primer OPA19), and four additions (1.8-, 0.71-, 2.0- and 1.15-kb fragments with the OPA 18, OPA 19, OPC 14, and OPE 12 primers, respectively) (Fig. 2). We performed RAPD-PCR analysis on three pools of DNA from kidney tissues of age-matched control animals, each pool containing DNA from two animals. Using the above sets of primers, we did not observe any change in the RAPD-PCR fingerprints between the different batches of untreated controls (data not shown). This indicates that there were no spontaneous mutations at these loci among the control animals, or at least that the frequency was too low to be detected by the analysis. This observation suggests that these mutations were induced as a result of DES

Table 2: Summary of the gains, losses and intensity changes in RAPD-amplified products from stilbene estrogen-induced kidney tumors
compared to age-matched controls. Fragment sizes of the amplification products are given in kilobases (kb) ¹ represents reduction in
intensity and ⁺¹ represents increase in the intensity.

Primer	Sequence (5' to 3')	Number of Amplification Products (no. Variable)	Size (Kb) of variable amplification – products in tumor DNA			
			Lost	Gained	Intensity Difference	
OPA 08	GTGACGTAGG	7 (5)	1.55 1.0, 0.54	0.75	0.55 ^{-I}	
OPA I I	CAATCGCCGT	7 (3)	I.5, .75	0.70	-	
OPA 15	TTCCGAACCC	5(1)	-	1.2	-	
OPA 17	GACCGCTTGT	5 (4)	-	0.45, 0.57	0.7 ⁺¹ , 0.62 ⁻¹	
OPA 18	AGGTGACCGT	4 (1)	-	1.8	-	
OPA 19	CAAACGTCGG	4 (2)	0.6	.71	-	
OPC 01	TTCGAGCCAG	5 (3)	0.3	0.6, 0.478	-	
OPC 09	CTCACCGTCC	5 (1)	-	2.0	-	
OPC 13	AAGCCTCGTC	6 (1)	1.95	-	-	
OPC 14	TGCGTGCTTG	4 (2)	1.1	2.0	-	
OPE 03	CCAGATGCAC	6 (6)	1.6, 0.7	2.0, 1.5, 1.2, 0.8	-	
OPE 12	TTATCGCCCC	11 (1)	-	1.15	-	
OPK 02	GTCTCCGCAA	3 (1)	0.3	-	-	
OPK 09	CCCTACCGAC	8 (3)	1.15	1.9, 1.35	-	
OPK 13	GGTTGTACCC	2 (1)	3.1	-	-	
OPK 16	GAGCGTCGAA	4 (2)	1.6, 1.0	-	-	
OP-26-07	TCGATACAGG	5 (2)	-	-	0.7 ⁻ , 1.5 ⁻	
OP-26-12	CTGCTTGATG	5 (2)	1.75	-	I.6 ^{-I}	
OP-26-25	GATCATAGCG	3 (3)	1.2	-	I.25 ^{-I} , 0.8 ^{-I}	

treatment and were not spontaneous mutations associated with the aging of animals.

Loci with mutations detected only in tumors and not in surrounding uninvolved tissue adjacent to tumors

Of the 44 mutated loci that were amplified, 37 (84.10%) harbored mutations only in tumor DNA and not in the adjacent uninvolved kidney tissue DNA, as determined through comparison with kidney DNA from age-matched control hamsters. Representative fingerprints are shown in Figs. 2,3,4,5. The sizes of 37 individual loci, and the sequence of the primers used to amplify them, are given in Table 2. Of these 37 mutated loci, Fig. 3 shows tumor DNA-specific losses in the 1.55-, 1.0-, 0.54-kbp products amplified with primer OPA 08, the 0.3-kbp fragment amplified with OPK02, the 1.15-kbp fragment amplified with OPK 09, and the 3.1-kb product amplified by OPK 13. Fig. 4 shows that losses of the 1.95-kbp product amplified using primer OPC 13, the 1.6- and 0.7-kbp fragments amplified with primer OPE 03, the 1.6-, and 1.0-kbp products amplified with primer OPK 16, the 1.75-kbp fragment produced with primer OP 26-12, and the 1.2kbp product amplified with primer OP 26-25 also were specific to reactions conducted with tumor DNA. In addition, loss of the 0.3-kbp product amplified with primer OPC 01 and the 1.1-kb fragment produced with OPC14 was observed in reactions conducted with tumor DNA and not with DNA from uninvolved (non-tumor) surrounding tissue adjacent to tumors (Table 2). The gain of 18 loci (0.75 kbp with OPA 08, 1.2 kbp with OPA 15, 1.9 and 1.35 kbp with OPK 09 [shown in Fig. 3], and 2.0, 1.5, 1.2 and 0.8 kbp with OPE 03 [shown in Fig. 4]) was observed in the tumor DNA as compared to control DNA. Out of the remaining eight loci, seven showed loss of intensity (the 0.55-kbp fragment amplified with primer OPA 08 that is shown in Fig. 3; and the 0.7- and 1.5-kbp fragment amplified with primer OPA 08 that is shown in Fig. 3; and the 0.7- and 1.5-kbp fragment amplified with primer OP26-12, and 1.25- and 0.8-kbp fragments produced with primer OP 26-25 shown in Fig. 4), and one showed a gain of intensity (the 0.7-kb produced amplified with primer OPA 17) compared to data from age-matched control kidneys.

Loci with mutations at restriction enzyme recognition sites

Primers that did not reveal differences in fingerprints generated using undigested control and tumor DNAs were subjected to RAPD-PCR analyses with DNAs digested with *Alu I, EcoRI, BamHI, HindIII,* and *HinfI.* Only in *Alu I*digested tumor DNA we observed a loss of 0.3-kbp locus and gain of 0.478- and 0.6-kbp loci with primer OPC01 (Fig. 5). Similarly, a gain of 0.45-kbp and 0.57-kbp fragments was found for *Alu I-*digested tumor DNA with primer OPA17 (Fig. 5). Also, *Alu I-*digested tumor DNA produced a reduction in the intensity of the 0.65-kbp



Representative RAPD fingerprints showing gains/losses and intensity differences for amplification products indicated by arrow with size of the amplified fragment (kb) common to DNA from both stilbene estrogen-induced kidney tumors (T) and uninvolved surrounding tissue adjacent to tumors, i.e., free of macroscopic tumors (M), in comparison with DNA from agematched control kidneys (C). Primers used (OPAII, OPA 18, OPAI9, OPC 14 and OPEI2) are indicated at the bottom of each set of fingerprints.

fragment and a gain in intensity of the 0.7-kbp product amplified with primer OPA17 (Fig. 5). These loci presumably harbored mutations in the *Alu I* restriction enzyme recognition sequence.

Characterization of a mutated locus

The 478 bp RAPD amplification product amplified with primer OPC01 was further cloned and sequenced. Sequence homology searches revealed that part of the fragment's sequence (from nucleotide 122 to 252) had significant similarity (85% identity) with the Syrian hamster gene for Cytochrome P-450 IAI (Fig. 6).

Discussion

This is the first attempt at a comprehensive analysis of mutations in stilbene estrogen-induced Syrian hamster kidney tumors. We screened 476 kbp of the hamster genome in 365 amplified loci. The important finding that emerged from this study is that the DNA of stilbene estrogen-induced tumors showed both qualitative (gains/ losses presumably as a result of point mutations or length mutations) and quantitative changes (intensity changes presumably as a result of aneuploidy or polyploidy) in RAPD-PCR fingerprints. RAPD-PCR fingerprint changes also were seen in DNA from non-tumor kidney tissue adjacent to tumors in DES-treated hamsters and these changes matched changes found in DNA from the tumors. This suggest that these mutations occurred in early stages of stilbene estrogen-induced renal carcinogenesis. In addition, one of the mutated loci had significant sequence homology with the hamster *CypIAI* gene.

Exposure of hamsters to DES and E_2 results in aneuploidy, chromosome aberrations, and DNA stand breaks in renal cells [7,8]. However, these observations provide no information about specific DNA sequences or gene loci that have undergone change. Other studies have uncovered very few DNA sequence changes in DES-induced tumors, including previous attempts at screening for changes [26,34,28]. RAPD-PCR is a unique method for identifying specific losses and gains of chromosomal regions associated with stilbene estrogen tumorigenesis. The results of this study demonstrate the ability of the RAPD-PCR to detect and isolate, in a single step, the



Representative RAPD fingerprints showing gains/losses and intensity differences of amplification products indicated by arrow with size of the amplified fragment (kb) for DNA from stilbene estrogen-induced kidney tumors (T), uninvolved surrounding tissue adjacent to tumors, i.e., free of macroscopic tumors (M), and age-matched controls (C). Primers used (OPA 08, OPA 15, OPK 02, OPK 09, and OPK 13) are indicated at the bottom of each set of fingerprints.

genetic alterations in stilbene estrogen-induced cancer cells, including alterations presumably caused by loss of heterozygosity, and homozygous deletion or insertion mutations. RAPD-PCR demonstrated both qualitative and quantitative changes in DES-induced tumors, which provides the basis for an alternative molecular approach for studying cancer cytogenetics that is applicable to stilbene estrogen-induced tumors from humans as well as experimental models. Thus, RAPD-PCR may be useful for searching for alterations in archived samples from women with stilbene estrogen-linked cancers and in those individuals who are at high risk of developing stilbene estrogen exposure-associated cancer.

Although RAPD-PCR provides no direct information on the functional importance of the mutated loci, analysis of these genetically-altered loci may provide suggestive evidence for loci that participate in tumorigenesis. Cloning and sequencing the amplified loci detected in this study revealed that one of the mutations is in a coding region of the genome. The sequence of the 478-bp RAPD product amplified by the OPC01 primer had significant homology with the *Cyp1A1* gene from Syrian hamster. Since Cyp1A1 may be involved in the metabolism of DES to genotoxic derivatives (see below), amplification of this sequence could have a functional significance in stilbene estrogeninduced carcinogenesis. Our present findings indicate that novel gene(s) susceptible to mutation as a result of stilbene estrogen treatment can be identified using this RAPD screening method. Further characterization of additional target sites may lead to the identification of additional genes susceptible to mutation and provide clues as to the mechanism of DES-induced tumorigenesis.

Previous studies demonstrated that both synthetic and natural steroidal estrogens are negative in classical bacterial and mammalian gene mutation assays [35,36]. This led to the conclusion that estrogen is a nongenotoxic and nonmutagenic chemical [10,37,38], and estrogen was, therefore, classified as an epigenetic carcinogen [10]. More recently, mutations have been detected in estrogen-induced kidney tumors: in the catalytic domain of an important DNA repair enzyme, DNA polymerase ß, and in microsatellite repeats [26,28]. These mutations could be due to genomic instability caused by tumor progression, rather than directly due to estrogen-induced genotoxicity.

Other studies, however, suggest that estrogens (or estrogen metabolites) are genotoxic (see Introduction). DES and E_2 (after conversion to catechol E_2) are oxidized to quinone primarily by cytochrome P450 1A1 [39]. DES or



Representative RAPD fingerprints showing gains/losses and intensity differences of amplification products indicated by arrow with size of amplified fragment (kb) for DNA from stilbene estrogen-induced kidney tumors (T) and age-matched controls (C). Primers used (OPC 13, OPE 03, OPK 16, OP 26-07, OP 26-12, and OP 26-25) are indicated at the bottom of each set of fingerprints.



Figure 5

Representative RAPD fingerprints for *Alu I*-digested and undigested DNA from stilbene estrogen-induced kidney tumors (T) and age-matched control (C) tissue. Arrows indicate the RAPD loci harboring mutations in the *Alu I* restriction enzyme recognition sequence. Note that these mutations were not detected in the RAPDs from undigested DNA using the same primers.

estradiol semiquinone also is formed as an intermediate in the process of oxidation of stilbene estrogen or catechol estrogens. The semiquinones react with molecular oxygen and generate superoxide, which is further reduced to the hydroxyl radical. Various types of DNA damage are produced by either estrogen metabolites or free radicals binding covalently to DNA [11-13,16,17]. Recent studies demonstrate the genotoxicity of DES quinone [23,25] and both DES and E2 are mutagenic in the gpt+ Chinese hamster G12 cell line [24]. In vivo formation of stilbene estrogen quinone, covalent binding of DES to nuclear DNA [14], and the chromosomal abnormalities observed in the kidneys of DES-treated Syrian hamsters [7,8] are consistent with a mechanism of DES-induced DNA damage. Thus, the ability of DES reactive metabolites or free radicals formed during the redox cycling of DES to covalently modify bases of DNA, arrest the gene replication and produce mutations suggests that these reactive intermediates could initiate and promote the formation of DES-induced kidneys tumors.

Mutation is a largely random phenomenon, and its targets can be relatively nonspecific. Any mutational analysis designed to screen mutation in a particular gene or genomic region may not detect mutations produced by an otherwise mutagenic carcinogen. This may explain why

1	ttcgaggcca	gccttttaga	tctctcagtt	cccctttact	ttcctgcgac	ctcagtggat	
61	caggcgaaag	actggagatt	ttttatttta	tttcttattt	tacctttttt	tttttttt	
121	ggagaatggg	tttctctcta	tccttttgga	ggctgtcctg	gaactggctc	tatagaccac	
181	ggatggcctc	catcctacct	acctctgcct	ccggagcgtt	gggattaaag	gcttgcgcca	
241	ccaacatctg	gctctctgaa	aatcactaac	gaacatcctc	cctatctggg	acatttaagc	
301	ctccctcagt	aatgaaccat	gggctcactc	gttcaatcca	ttttaaaaaa	gcctttcatg	
361	gttttagtcc	actgttcttc	aagtaacttc	ttttatgtga	tactgcatct	aaaacttcta	A
421	ggggactatt	tcccatcttt	tcttttccta	ttttccccca	tccatccctg	ggctcgaa	IX.
AP-PCR prod	uct 122 GAGA	ATGGGTTTCTCT	CTATCCTTTTGG	AGGCTGTCCTG	GAACTGGCTCTA	FAGACCACG 181	
CYP P4501	Al 3239 GAGA	CAGGGTTTCTCT	GTATTGTTTTGG	aggetgtetet	AAACTCGCTCTG	TAGACCAGG 3298	
AP-PCR pr	oduct 195 CTA	CCTACCTCTGCC	TCCGGAGCGTT	GGGATTAAAGGC	TTGCGCCACCAA	CATCTGGC 252	
CYPP4501	Al 3321 CTG	CCTGCCTCTGCC	TCCCCAGTGCT	GGGATTAAAGGT	GTGCACCACCAA	CACCCGGC 3378	_
							В

Sequence (**A**, upper panel) of the 478-bp product amplified with primer OPC 01 and *Alu I*-digested DNA from a stilbene estrogen-induced hamster kidney tumor. The homology with Syrian hamster gene for Cytochrome P-450 IAI is shown in **B** (lower panel)

many previous studies have failed to detect estrogeninduced mutation. Thus, the screening of mutations at a genome-wide level with RAPD-PCR, which is not biased to any particular gene/genomic region, may be a better approach for drawing conclusions about the mutagenic potential of a chemical. RAPD-PCR by itself does not distinguish between initiating lesions induced by genotoxic carcinogens and mutations generated during tumor progression as a consequence of genomic instability. However, the identification of mutations common to both stilbene estrogen-induced tumors and the uninvolved (non-tumor) surrounding kidney tissues adjacent to tumors strongly suggests that stilbene estrogen treatment may be responsible for some of the early mutations in the genome of Syrian hamster kidney.

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