Frequent and Specific Mutations of the Rat p53 Gene in Hepatocarcinomas Induced by Tamoxifen

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ABSTRACT

Tamoxifen (TAM) is a triphenylethylene antiestrogen used for the treatment, and in clinical trials for the prevention, of breast cancer in women. In rats, TAM is a strong liver carcinogen which induces the formation of liver DNA adducts. The DNA of 24 hepatocarcinomas (HCCs) collected at necropsy from individual female Sprague-Dawley rats that were given 22.6 mg/kg TAM daily for 12 months was studied for the presence of mutations in exons 5–9 of the p53 gene by single-strand conformation polymorphism and DNA sequencing analysis. The sequences of introns 5–8 of the rat p53 gene were determined in order to design primers homologous to regions located in these introns. p53 mutations were found in 50% (12 of 24) of the HCCs. These mutations were all specifically clustered in two sites, codons 231 (exon 6—7) and 294 (exon 8). Nine HCCs contained a transition from adenine to guanine in the second base of codon 231 (CAC to CGC), which resulted in a histidine to arginine amino acid substitution; 4 HCCs contained a nonmiscoding transition from cysteine to cysteine in the third base of codon 294 (TGC to TGT; cysteine to cysteine). One HCC contained both mutations. The present report supports previous observations on the genotoxicity of TAM in rodents and raises concerns about its use as a chemopreventive agent against breast cancer in women.

INTRODUCTION

TAM2 is a triphenylethylene antiestrogen widely used in the treatment of breast cancer in women (1) and in clinical trials as a preventive agent against the same disease (2). TAM displays a low level of short-term adverse effects in human beings (3). However, several independent studies performed on laboratory animals have demonstrated that TAM is a strong liver carcinogen in rats of both sexes (4–6) and causes the formation of adducted bases in the livers of male and female rats (7–9), female mice (7), and female hamsters (10), providing a genotoxic basis for the observed carcinogenicity. Furthermore, in humans, recent clinical studies have also attracted attention to the carcinogenic potential of TAM on the uterus and liver (11). In vitro experiments have demonstrated that reactive species were generated during hepatic metabolism of TAM (12, 13) by mono-oxygenases (13).

The p53 tumor suppressor gene is a central element in the regulation of the cell cycle (14, 15). It is reportedly the most frequently mutated gene in human tumors (15). In humans, specific patterns of mutations in the p53 gene exist and have been matched anatomically with neoplasms sites (16) and etiologically with exposure to aflatoxin B1 in HCCs of patients in regions of Africa and China (17, 18). To characterize the genotoxic effect of TAM on the p53 tumor suppressor gene, we obtained 24 HCCs from individual female rats treated with TAM (9). Prior to p53 analysis, the sequences of exons 5–8 of the rat p53 gene were determined in order to design primers located in these exons and 7 and 8 of the p53 gene in these TAM-induced HCCs.

MATERIAL AND METHODS

Determination of the Genomic Sequence of Rat p53 Introns 5–8. PCR was performed with 0.5 μg of normal liver genomic DNA, 25 pmol of primers (IDT, Coralville, IA) homologous to intron 4 and exon 9 of the rat p53 gene (Table 1), 40 nmol of each deoxynucleoside triphosphate, and 1 unit of Vent(R) DNA polymerase (New England Biolabs, Beverly, MA) in the manufacturer’s 2.0 mm magnesium sulfate buffer for 35 cycles of amplification through denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 1 min) on an Ericomp Thermal Cycler (Ericomp, San Diego, CA). Multiple amplicons (approximate length, 1.1 kilobases) were cloned into pCR vectors (T/A cloning kit; Invitrogen, San Diego, CA) and sequenced using primers (Table 1) homologous to regions of exons 5–9 and a Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, OH) according to the manufacturer’s protocols. The samples were resolved on 8% acrylamide–7 M urea gels. Autoradiography was performed at room temperature for 72 h with 1 intensifying screen.

Origin of Tumors and DNA Extraction. Twenty-four female SD rats were administered 22.6 mg/kg TAM daily for 12 months (9). At the time of sacrifice, 24 HCCs were collected from individual rats, immediately frozen in liquid nitrogen, and stored at −80°C. A representative sample of each tumor was processed for routine histopathological examination, stained with hematoxylin and eosin, and examined by light microscopy. All tumors were diagnosed as HCCs and in all cases, tumor cells constituted over 80% of the cellular population. DNA was extracted from the HCCs according to the method of Sambrook et al. (19) and stored at −20°C in Tris-EDTA (10 mM Tris, pH 8.0–1 mM EDTA). DNA from the livers of 2 untreated SD rats were similarly prepared as negative controls.

PCR Amplification and SSCP Analysis. Three pairs of primers homologous to regions located in introns 4–8 and exon 9 of the rat p53 gene were used in PCR of individual exons 5–9 (Table 1). PCR was performed essentially as above with 0.5 μg of genomic liver DNA and 0.5 μl of [α-32P]dCTP (3000 Ci/mmol, 10 μCi/ml; Amersham, Chicago, IL) for 35 cycles of amplification through denaturation (94°C, 1 min), annealing (1 min at 52°C for exon 5, 65°C for exon 6–7, and 56°C for exons 8–9), and extension (72°C, 1 min). The PCR products were run with molecular weight markers on 5% polyacrylamide gels; the appropriate bands (328, 294, and 262 base pairs for primers 5–9) were excised from the gel. The DNA extracts were then denatured at 100°C for 3 min prior to loading on 2% agarose in 0.5 μM EDTA, pH 8.0. The samples were then electrophoresed for 90 min at 100 V. The gels were then cut into 3 mm sections, blotted onto nylon membranes, and subjected to Southern blotting analysis with a 32P-labeled oligonucleotide probe specific for the p53 gene.

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2 The abbreviations used are: TAM, tamoxifen; HCC, hepatocarcinoma; PCR, polymerase chain reaction; SD, Sprague-Dawley; SSCP, single-strand conformation polymorphism.

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concentrated by lyophilization and electrophoresed in 1.5% low melting point agarase. The bands were excised from the agarase gel and the DNA was sequenced according to the method of Anderson et al. (20) using a Sequenase 2.0 kit and [α-35S]dATP (3000 Ci/mmol, 10 mCi/ml) and resolved as above. The primers used are shown in Table 1. Both DNA strands of each shifted band and of cloned wild-type p53 DNA were sequenced. The complementary DNA sequence published by Soussi et al. (21) was used as a reference.

RESULTS

The sequence of the rat p53 gene introns 5–8 was determined. PCR primers homologous to intronic regions of the p53 gene were used to exclude p53 pseudogene amplification because at least two p53 pseudogenes have been identified in the rat genome (22, 23) and PCR performed with primers homologous to exonic sequences amplifies the gene and pseudogenes. The genomic sequences of the p53 introns 5–8 from SD rats are presented in Fig. 1. No intron separates exons 6 and 7, which is different from the human (24) and mouse (25) genomic p53 sequences and confirms a recent report by Hulla and Schneider (22). Introns 5, 7, and 8 measure 78, 309, and 78 base pairs, respectively, and are 3, 39, and 13 base pairs shorter, respectively, than their human counterparts. The intronic regions flanking each exon have been described previously (22). The present report confirms them and provides the precise base sequences contained in introns 5–8.

Fifty % (12 of 24) of the analyzed HCCs contained p53 mutations in the highly conserved region encompassing exons 5–9 (Figs. 2 and 3). All observed mutations were clustered specifically in two sites, codons 231 (exon 6–7) (Fig. 4) and 294 (exon 8), and resulted in miscoding mutations in 37% of the HCCs. In the 9 HCCs mutated in exon 7, these mutations consisted of a transition from adenine to guanine in the second base of codon 231 (CAC to CGC), which resulted in an amino acid substitution from histidine to arginine. In the 4 HCCs mutated in exon 8, a nonmiscoding transition from cytosine to thymidine in the third base of codon 294 (TGC to TGT; cysteine to cysteine) was observed. One HCC (110–1) contained both mutations.

Wild-type p53 was retained in all samples and, in most cases, its autoradiographic intensity was stronger than that of the SSCP-shifted band as assessed visually on the SSCP autoradiographic pattern (Figs. 2 and 3). Only one HCC sample mutated in codon 294 (Fig. 3) (101–1) contained an approximately equal amount of wild-type and SSCP-shifted DNA.

DISCUSSION

TAM is a strong liver carcinogen in rats (4–6, 9), in which it has been shown to cause DNA adducts in the liver of males (8) and females (7, 8, 10). The present study demonstrates the presence of a high frequency (50%) of p53 mutations in HCCs induced by TAM in female SD rats. These mutations selectively affected two sites of the p53 gene and resulted in a miscoding mutation in 37% of the HCCs. The discovery of specific mutations in HCCs from rats treated with...
RAT p53 MUTATIONS IN HCCS INDUCED BY TAM

Fig. 3. SSCP analysis of exons 8 and 9 of the rat p53 gene. pRS3 5/9, cloned wild-type p53; pRS5/9-M 882, cloned mutated p53; NI, unexposed livers; 73-3 to 125-1, HCCs. Arrows, shifted bands.

(17) but were not found in HCCs from rats treated with this compound (26). Smith et al. (27) did not identify p53 mutations in HCCs from rats given BR931, a peroxisome proliferator. In another study, the same authors (28) showed the presence of p53 point mutations in 62% of the HCCs from rats fed a choline-devoid diet but did not demonstrate a clustering of the mutations at a specific site.

Even if only a limited number of studies of p53 gene mutations have been performed on rat tissues thus far, our report, together with a study by Ohgaki et al. (29) describing selective mutations of codons 204 and 213 in renal tumors after administration of alkylating N-nitroso compounds, raises the exciting possibility that the exposure of animals to a specific agent could help identify specific mutations induced by this agent. Human tumoral tissues could then be screened for the presence of specific mutations ascribable to definite agents.

Cases of hepatic (11) and uterine (11, 30–32) tumors after TAM administration have been reported in humans. The screening of the human p53 gene in tumoral tissues from women exposed to TAM could reveal the presence of mutations comparable to those observed in rats in the present study and could enable us to ascertain the relevance of the genotoxicity of TAM to human exposure.

Heterogeneity of p53 mutations according to the histological grade and the position of the lesion within the same hepatic tumor has been demonstrated in humans (33). In the present study, shifted SSCP bands displayed weaker signals than their wild-type counterparts in all but one sample. One tumor contained two different p53 mutations. These observations imply that only a percentage of the tumor cells carried p53 mutations and strongly suggest that the observed mutations were somatic and specific to the analyzed tissues. However, because normal tissues from the affected animals were unavailable at the time of the study and because we are not aware of other p53 mutation analyses performed on SD rats, we cannot formally exclude the possibility that these mutations are the manifestation of a polymorphism specific to this particular strain of rats. The mutations observed in codon 294 reflect the sensitivity of a specific DNA site affected early in HCCs induced by TAM; however, because they are

TAM further buttresses the hypothesis that TAM is, or undergoes biotransformation into, a genotoxic metabolite in this species. Hard et al. (9) hypothesized that tamoxifen might be activated at the ethylene double bond, whereas toremifene, a structurally similar antiestrogen that differs from TAM only by the addition of a chlorine atom and does not induce HCCs, would be hindered from bioactivation by the presence of that chlorine atom.

We provide here additional information on the structure of the rat p53 gene. At least two p53 pseudogenes have been identified in the rat genome (22, 23). By using primers homologous to intronic sequences of introns 4–8, we have analyzed the p53 gene itself and circumvented the possibility of amplifying p53 pseudogenic sequences.

The present report is, to our knowledge, the first description of specific mutations of the p53 gene in rodent hepatic tissues. Codon 231 is located in an evolutionary highly conserved region of the p53 gene (15). Mutations affecting codon 249, a vicinal site, have been associated with aflatoxin B1 exposure in HCCs from human patients (17) but were not found in HCCs from rats treated with this compound (26). Smith et al. (27) did not identify p53 mutations in HCCs from rats given BR931, a peroxisome proliferator. In another study, the same authors (28) showed the presence of p53 point mutations in 62% of the HCCs from rats fed a choline-devoid diet but did not demonstrate a clustering of the mutations at a specific site.

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nonmiscoding, these mutations do not modify the biology of the tissue.

The presence of a high frequency of transitions from adenine to guanine suggests that in rats, the adenine adducts are important. This observation is not supported by a recent in vitro study by Pongracz et al. (34) demonstrating the preponderance of guanine adducts to guanine suggests that in rats, the adenine adducts are important. This observation is not supported by a recent in vitro study by Pongracz et al. (34) demonstrating the preponderance of 2 guanine adducts in rat liver tissue.

In conclusion, we have shown that a high frequency of mutations of the rat p53 gene is present in HCCs from rats exposed to TAM and that these mutations are specifically located at codons 231 and 294. These observations and a recent report of increased frequency of liver tumors in breast cancer patients treated with TAM (11) emphasize earlier concerns (9, 35) expressed about the use of TAM as a chemopreventive agent against breast cancer. Analysis of the p53 gene in tumors from patients treated with TAM could help answer the question of the genotoxicity of the drug in humans.

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REFERENCES


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