ABSTRACT

Previous studies have shown renal mesenchymal tumors (RMTs) induced in rats by a single intrarenal injection of nickel subsulfide and iron are more pleomorphic and metastatically aggressive than RMTs induced by a single ip injection of methyl(methoxymethyl)nitrosamine (DMN-OMe). While both RMT types contain high levels of K-ras activation, the specific mutational spectra within codon 12 of K-ras are quite different. Nickel subsulfide and iron-induced tumors exhibited codon 12 GGT→GT transitions exclusively, while DMN-OMe RMTs showed a wide array of codon 12 mutations, as well as mutations within codons 61 and 63. In rats with Ni3S2 + Fe ~ exhibited point mutations in exon 1 of K-ras, while DMN-OMe-induced tumors showed point mutations in exons 6 and were predominantly at two specific nucleotides, 610 (G>A) and 638 (G→A). Unexpectedly, in two of the eight tumors studied, both mutations were detected coincidentally, suggesting possible RMT p53 mutational “hot spots” similar to the codon 249 “hot spot” found in human hepatocellular carcinomas from persons living in certain high cancer risk areas (20–22).

INTRODUCTION

Much attention has been placed recently on understanding the role of p53 in carcinogenesis. The p53 gene codes for a nuclear protein whose functionality appears to be very important in negative regulation of the cell cycle (1, 2). The p53 protein has a half-life of approximately 6 to 20 min (8, 9). In contrast, some point mutated forms of the protein can exhibit half-lives of 1.5 to 12 h (8, 10) and subsequently accumulate intracellularly. The mutational events seem to cluster predominantly around five highly conserved amino acid domains which include portions of exons 1–7 (reviewed in Ref. 11). Deletions or insertions causing frame-shifts result in nonfunctional, truncated forms of the p53 protein. Mutations which destroy or create splicing sites in the gene can have similar protein-inactivating effects. The functionality of the wild-type p53 can also be altered by its interaction with binding proteins, such as heat shock protein 70 (12, 13) and the mdm-2 gene product (14, 15). To date, the loss of wild-type p53 function, or gain of a new function due to mutation, is one of the most commonly reported genetic changes in human cancer with an overall frequency of 50% (3, 16). In contrast, little is known about the role of p53 inactivation in experimental tumors from animal models. The presence of one or more p53 pseudogenes (ψ-genes) in the mouse (17) and the rat (18, 41) genomes complicates analysis of sequence changes at the DNA level in these species.

Recently, a report based on genomic DNA analysis has suggested that inactivation of p53 plays an important role in chemically induced renal tumorigenesis in the rat (19). RMTs induced in Wistar rats with N-nitrosodimethylamine reportedly exhibited a high incidence (75%) of point mutations within the p53 gene. The majority of these mutations were detected within the evolutionarily conserved regions of exon 6 and were predominantly at two specific nucleotides, 610 (G→A) and 638 (G→A). Unexpectedly, in two of the eight tumors studied, both mutations were detected coincidentally, suggesting possible RMT p53 mutational “hot spots” similar to the codon 249 “hot spot” found in human hepatocellular carcinomas from persons living in certain high cancer risk areas (20–22).

In an effort to further correlate specific molecular events with individual carcinogens, the present study was designed to compare the mutational spectra evoked in the rat p53 gene by DMN-OMe and Ni3S2 + Fe ~ in RMTs as detected by SSCP (23–25) and CCM (26–28). RMTs induced by these carcinogens differ in their K-ras mutational spectra as well as their metabolic potential. RMTs induced in rats with Ni3S2 + Fe ~ exhibited point mutations in exon 1 of K-ras, while DMN-OMe-induced tumors showed a wider range of mutations within exon 1 (codons 12 and 13) as well as within exon 2 (codons 61 and 63) (30). In addition to the dissimilar patterns of K-ras mutations demonstrated by these two initiating protocols, the biological behavior of the tumors induced was remarkably different. RMTs induced with Ni3S2 + Fe ~ were biologically very aggressive tumors which frequently metastasized to the lung. In contrast, RMTs induced by DMN-OMe rarely metastasized. These experimental circumstances, therefore, provide opportunities both to compare the spectrum of p53 mutations caused by carcinogens possessing different patterns of mutational activities and to establish to what degree specific p53 mutational events correlate with and perhaps contribute to the metastatic potential of these neoplasms.

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1 The abbreviations used are: RMT, renal mesenchymal tumor; Ni3S2 + Fe ~ nickel subsulfide and iron; DMN-OMe, methyl(methoxymethyl)nitrosamine; SSCP, single-strand conformation polymorphism; CCM, chemical cleavage of mismatches; HA, hydroxylamine; NMU, N-nitrosomethylurea; OT, osmium tetroxide; PCR, polymerase chain reaction; PIP, piperidine; RT-PCR, reverse transcription-polymerase chain reaction; TBE, tris-borate-EDTA; ψ-genes, pseudogenes; cDNA, complementary DNA.

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MATERIALS AND METHODS

Origin of Renal Mesenchymal Tumor Samples. RMTs were obtained from two separate studies (29). In the first study, 6-week-old male F344 rats received an intrarenal injection of an equimolar suspension of NiS2 (5.0 mg) and Fe3O4 powder (3.4 mg) in the cortex of the right kidney. Renal tumors began to appear by 24 weeks of age and were harvested over a 25-week period. The second type of RMTs was induced in male and female F344 rats by a single i.p. injection of DMN-OMe (4.0 mmol/kg body weight) 48 h after birth. Renal tumors began to appear by 26 weeks of age and were harvested during a 77-week period. At necropsy, sections of each tumor were fixed in 10% buffered formalin and were processed for light microscopy, while the remaining tumor mass was frozen in liquid nitrogen and stored at −70°C.

Oligonucleotide Primers. The following oligonucleotide primers were derived from the rat complete cDNA sequence (Genbank accession no. X13058; Ref. 31) and analyzed for compatibility with the Oligo primer analysis software (National Biosciences, Plymouth, MN). Their specific locations within the p53 gene are demonstrated in Fig. 1. Primer 1: 5'-AACITACCAAGGC-AACTATG-3'; Primer 2: 5'-CTCAGGTGGCTCATACGGTA-3'; Primer 3: 5'-AGGCTTGTCTGATGGTGAC-3'; Primer 4: 5'-CGGCGAACTCTCTTCTTTT-3'; Primer 5: 5'-TCTTGTTGTGGCTGCTCPC-3'; Primer 6: 5'-CTCITCTTCGTCTGCCTGAGC-3'.

RNA Preparation and RT-PCR. Total RNA was isolated from individual frozen tumors and normal kidneys with the guanidine isothiocyanate procedure (32). p53 mRNA, including the conserved coding regions of the gene, was specifically reverse-transcribed to cDNA with the use of a GeneAmp RNA PCR kit (Perkin Elmer, Norwalk, CT). An oligonucleotide (0.75 μM; primer 6; Fig. 1) complementary to the 3' end of the p53 mRNA was incubated in a 20-μl reaction mixture containing sample total RNA (0.5 μg), MgCl2 (5 mM), Tris (pH 8.3; 10 mM), KCl (50 mM), dNTPs (1 mM), and Moloney murine leukemia virus reverse transcriptase (50 units). Incubation conditions were as follows: 45 minutes at 42°C, 5 minutes at 99°C, and 5 minutes at 4°C. An 817-base pair product was then amplified in a 100-μl reaction utilizing the 20 μl of eDNA as a template under the following conditions: MgCl2 (2 mM), Tris (pH 8.3; 10 mM), KCl (50 mM), dNTPs (0.2 mM), Taq polymerase (2.5 units), and an upstream primer (0.15 μM; primer 1; Fig. 1). PCR was performed for 40 cycles with 1 min at 92°C and for 1 min at 50°C. The 817-base pair fragments were gel-purified and included exons 4-10. It was necessary to generate smaller fragments for analysis due to the inherent limitations of the SSCP method. Three overlapping fragments (370, 305, and 303-base pair products) were amplified using primers 1 and 2 to yield a 370-base pair product (exons 4-6); primers 3 and 4 to yield a 353-base pair product (exons 5-8); and primers 5 and 6 to yield a 305-base pair product (exons 8-10). The PCR reaction mixture, composed of MgCl2 (1.5 mM), Tris (pH 8.3; 10 mM), KCl (50 mM), dNTPs (0.2 mM), and Taq polymerase (2.5 units), DNA template (~1 μg), and [32P]-end-labeled primers (0.15 μM) was incubated at 92°C for 45 s and 50°C for 45 s for 30 cycles. The [32P]-labeled PCR fragments (3 μl) were diluted with 26 μl of stop solution (95% formamide-20 mM EDTA-0.05% bromphenol blue-0.05% xylene cyanol FF) and 1 μl of methylmercury hydroxide (1 μ) as described (33). Samples were then heated to 98°C for 5 min and placed on ice, followed by electrophoresis (1 μl/well) on a 0.5% Hydrolik Mutation Detection Enhancement gel (AT Biochem, Malvern, PA) with or without 5% glycerol at a constant 8 watts for 18 to 24 h at room temperature in 0.6x TBE. The gels were dried on filter paper and exposed to X-ray film at −80°C in the presence of an intensifying screen.

Detection of p53 Point Mutations By CCM Analysis. Detection of point mutations by CCM was carried out essentially according to Grompe et al. (26). The labeled wild-type 817-base pair probe strands were generated by additional amplification steps using 0.15 pmol of [32P]-end-labeled primer and 0.15 pmol of cold opposite end primer. The conditions for the secondary amplifications were the same as those employed initially. Two reactions were done using one of the other radioactive primer (1 or 6), generating two labeled fragments specific for the sense and antisense strands. They were purified by elution after polyacrylamide gel electrophoresis. The wild-type probe (100,000 cpm; ~10-15 ng) was used to form a heteroduplex in the presence of a 10-fold excess (~150 ng) of the unlabeled tumor PCR products in annealing buffer (0.3 M NaCl, 3.5 mM MgCl2, 3 mM Tris, pH 7.5). The mixture was boiled for 5 min, annealed for 2 h or overnight, and then precipitated with ethanol, vacuum-dried, and dissolved in 20 μl of H2O. A heteroduplex mixture (6.5 μl) was used for each modification reaction. Chemical modifications and subsequent cleavage reactions were performed for each fragment. For the HA modification, which detects mismatched cytosine residues, the heteroduplexes were incubated in a 2.5 μl solution for 60 min at 37°C. For the OT modification, which detects mismatched thymidines, the heteroduplexes were incubated in an 0.8% (w/v) OT solution for 20 min at 37°C. The fragments were precipitated, cleaved with 1 X PIP for 30 min at 90°C, and analyzed by electrophoresis on a 5% acrylamide-8 M urea gel in 1 X TBE. The gels were dried on filter paper and exposed to X-ray film at −80°C in the presence of an intensifying screen. By using both types of modification reactions on each strand separately, all possible mismatches may thus be theoretically detected.

Detection of p53 Point Mutations By Direct Sequencing Analysis. When either SSCP or CCM analysis indicated that a p53 cDNA fragment contained a mutation(s), the corresponding 817-base pair gel-purified fragment was utilized as a template for direct dideoxy sequencing. The sequencing primers were chosen from those used to generate the three nested PCR fragments. The sequencing reactions were performed using [32P]-end-labeled primers and the Sequenase (version 2.0) kit (U.S. Biochemical Corp., Cleveland, OH) according to the manufacturer's instructions. Samples were loaded and electrophoresed on 8% polyacrylamide gels containing 50% w/v urea in 1 X TBE. The gels were dried on filter paper and exposed to X-ray film at −80°C in the presence of an intensifying screen.

RESULTS

Detection of p53 Point Mutations by SSCP Analysis. An 817-base pair cDNA product was amplified from 10 tumors of each type and from normal kidney. This product corresponded to nucleotide positions 297 through 1113 (codons 99-371) of the coding sequence and included exons 4-10. It was necessary to generate smaller fragments for analysis due to the inherent limitations of the SSCP method to detect mutations efficiently in fragments larger than roughly 400 base pairs (34). Additionally, to insure that mutations underlying primer sequences were not missed, three overlapping fragments (370, 353, and 305 base pairs) were amplified using the gel-purified 817-base pair cDNA fragment as a template. A rat bladder carcinoma cell line, ASU6 (provided by Dr. Makoto Asamato), which contains a point mutation within this 817-base pair region in codon 191 (CAT→AAT) of exon 6, was used as a positive control in this study. The particular spacing of this mutation in the gene conveniently locates it within two of the three overlapping PCR fragments (Fig. 1). Gel conditions with and without glycerol were used to ensure the highest degree of sensitivity (23).

None (0 of 10) of the 370-base pair or 353-base pair PCR fragments derived from the DMN-OMe-induced RMTs demonstrated conformational band shifts under any of the gel conditions used (Table 1). In contrast, 1 of the 10 DMN-OMe tumors exhibited a mobility shift in the 305-base pair fragment, corresponding to exons 8–10 (Table 1; Fig. 2). In addition to the shifted band, a band of equal intensity representative of the normal allele was also present. PCR fragments
Table 1 Incidence of p53 point mutations in rat renal mesenchymal tumors as detected by SSCP and CCM analyses

<table>
<thead>
<tr>
<th>Tissue/carcinogen</th>
<th>No. of cases</th>
<th>SSCP</th>
<th>CCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMTs/DMN-OMe</td>
<td>10</td>
<td>0/10</td>
<td>1/10</td>
</tr>
<tr>
<td>RMTs/Ni3S2 + Fe⁰</td>
<td>10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Normal kidney/none</td>
<td>3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

*bp, base pair.

Fig. 2. SSCP analysis of the 305 and 353 base pair fragments from normal kidney (Lane 1), ASU6 cell line (Lane 2), and 3 DMN-OMe-induced RMTs (Lanes 3, 4, and 5). In addition to the two bands representing the normal allele (Lane 1), an additional band or a band shift can be seen in Lane 4 of the 305-base pair fragment (a DMN-OMe RMT) and in Lane 2 of the 353-base pair fragment (ASU6-positive control).

Detection of p53 Point Mutations by CCM Analysis. Cleavage following OT modification of p53 heteroduplexes from ASU6 cells yielded a 274-base pair fragment corresponding to the size predicted by the position of the codon 191 mutation (data not shown). PIP treatment of HA-modified ASU6 heteroduplexes generated a fragment of the same size (274 base pairs). This is consistent with the nature of the mutation (C to A transversion), which on generation of a heteroduplex creates both a mismatched C (a target for HA) and a mismatched T (a target for OT). There was no specific cleavage of the unmodified ASU6 heteroduplexes.

CCM was also employed to detect single-base mutations in RT-PCR-amplified p53 mRNA derived from DMN-OMe and Ni3S2 + Fe⁰-induced RMTs. In the one SSCP-positive DMN-OMe RMT, PIP cleavage after HA modification generated a 738-base pair band not present after modification and cleavage of either the wild-type cDNA homoduplex or any of the other RMT heteroduplexes. This confirmed the existence of the point mutation in this sample. Chemical cleavage was not observed after OT or HA reaction in any of the remaining RMTs (data not shown).

Detection of p53 Point Mutations By Direct Sequencing Analysis. Confirmation of the p53 point mutation detected in the SSCP- and CCM-“positive” DMN-OMe-induced tumor is depicted in Fig. 3. The gel-purified 817-base pair fragment was used as a sequencing template and sequenced with the anti-sense oligomer #6. Direct sequencing detected a missense GCC→GAC transition mutation in codon 345 of exon 10 (Fig. 3), resulting in the replacement of an alanine residue by valine.

DISCUSSION

The present study evaluated the presence of p53 point mutations in rat RMTs induced by either a nitrosamine (DMN-OMe) or a metallic carcinogen (Ni3S2 + Fe⁰). An 817-base pair RT-PCR fragment from normal and tumor tissue, consisting of a p53 coding sequence within exons 4–10, was evaluated for point mutations by SSCP and CCM analyses. We found that only 10% (1 of 10) of the DMN-OMe-induced RMTs contained a point mutation detectable by these methods within this region of the gene, while the Ni3S2 + Fe⁰-induced RMTs exhibited no detectable mutations (0 of 10). The biological relevance of the detected mutation (GCC→GAC in codon 345 of exon 10) is unclear as it leads to substitution of a valine for an alanine, both of which are aliphatic/hydrophobic residues and functionally similar. Mutations in this region are rare in human tumors (11). The highly charged basic region at the carboxy terminus of the p53 protein does, however, contain the DNA binding domain (35), and altered binding capacities might conceivably result from a mutation of this type.
Previous studies have demonstrated that the RMTs induced by DMN-OMe and by Ni$_3$S$_2$ + Fe$^{6+}$, while both mesenchymal in origin, are very different tumors. Ni$_3$S$_2$ + Fe$^{6+}$-induced RMTs have been frequently shown to contain mutations within the 12th codon (exon 1) of K-ras that are exclusively GGT $\rightarrow$ GTT transversions (29). In contrast, DMN-OMe-induced RMTs were shown to contain K-ras codon 12 mutations which presented a more varied mutational spectrum. In addition, DMN-OMe RMTs contained mutations in exon 2 of K-ras within codons 61 and 63 (30). Differences in the properties of the two types of RMTs were also apparent. DMN-OMe-induced renal tumors grew to a large size but rarely metastasized. Conversely, Ni$_3$S$_2$ + Fe$^{6+}$-induced tumors were more malignant and metastasized to lung early in the tumorigenic process. The results of the present study, however, suggest that these significant differences in tumor phenotype are not the result of the presence or absence of mutations induced by the specific carcinogens in the conserved coding regions of the p53 gene. This implies that mutation of the p53 gene, at least in coding regions and yielding a gene product of altered amino acid sequence, is not a major pathway for tumor progression utilized by either of these two RMT types. The present results in the rat RMTs are consistent with previous studies that describe a low frequency of p53 mutations in renal cell carcinomas in humans (1 of 23; 4%; Ref. 36). In contrast, cultured human renal epithelial cells immortalized with dimethylbenz[a]anthracene, or N-hydroxy-2-acetylaminofluorene did not exhibit any mutational events within the conserved regions of the p53 gene (38, 39). Similarly, transgenic mice deficient for p53 develop normally but demonstrate an increased susceptibility to certain spontaneous cancers (40), most commonly lymphomas and sarcomas rather than the primary epithelial neoplasms of lung and liver that are characteristic of this species. This implies that the p53 product is not essential for embryonic development, but its absence predisposed the animals to spontaneous tumorigenesis selectively in certain tissues. Therefore, the inactivated form of the p53 protein is not universally required for tumor progression, and alternative pathways for tumor progression must exist.

The low frequency of p53 mutations exhibited by the DMN-OMe tumors in this study appear to be in direct contrast to the results obtained by Ohgaki et al. (19) in RMTs induced by another nitrosamine derivative, N-nitrosodimethyamine, in Wistar rats. Those RMTs were reported to contain a 75% (6 of 8) incidence of p53 mutations which were preferentially clustered within exon 6 at codons 204 and 213. In 2 of 8 RMTs (25%), both mutations were present simultaneously. That study used genomic DNA as a template for exon-based primers to amplify the rodent p53 gene from genomic DNA in our study. A complication of utilizing such exon-derived primers is the use of RT-PCR amplification of purified mRNA in our study. A complication of utilizing such exon-derived primers to amplify the rodent p53 gene from genomic DNA is the presence of p53 $\psi$-genes in mice (17) and rats (18, 41), specific segments of which can be amplified during PCR. We have deposited the sequence of one such processed rat $\psi$-gene that contains the aberrant “mutant” codon 213 sequence in GenBank as accession no. L12046. Polymorphisms between the “true” p53 gene and one or more of the highly homologous $\psi$-genes may then be misidentified as point mutations and become sources of misinterpretation. Fortunately, the $\psi$-gene contamination errors can be avoided by constructing PCR primers from species-specific intron sequences (42). In human p53 studies, exon-derived primers do not appear to yield such problems since no p53 $\psi$-genes have been identified in humans. However, disregarding these potential problems, the results by Ohgaki et al. (19) could represent carcinogen-specific pathways of RMT tumorigenesis which may involve the p53 gene or interstrain differences among rats, as opposed to pathways that do not require p53 inactivation (i.e., N-nitrosodimethyamine versus DMN-OMe or Ni$_3$S$_2$ + Fe$^{6+}$).

Other possibilities exist that could result in indirect inactivation of the wild-type p53 protein without deletions, insertions, or point mutation within coding regions: (a) alternative posttranslational mechanisms such as p53 phosphorylation could indirectly modulate p53 function. Qualitative and quantitative differences in the levels of p53 phosphorylation have been found between normal and SV40-transformed rat cells (43); (b) combined effects of other inactivated tumor suppressor genes with those of activated oncogenes (e.g., K-ras in RMTs) may result in tumorigenesis even if the p53 gene product is normal (44); (c) mutations may occur in secondary genes whose products interact and transregulate p53. The major oncogene products of several viruses, including SV40, certain adenoviruses, and some strains of human papilloma viruses, bind to normal p53 and cause functional inactivation and potentially tumorigenesis (45–47). Similarly, the recently described cellular oncogene product of the mdm-2 gene tightly binds both wild-type and mutant p53 and is amplified in many human tumors in which p53 mutations were expected but not found (14, 15). Also implicated are the hsp70 proteins (12, 13) and the protein kinases, p34cdc2 kinase and casein kinase II (48–50). This body of evidence suggests that mutations upstream and downstream of the p53 signaling pathways can operationally substitute for mutations within the p53 gene itself. It is possible that it is these pathways that are the target in the two tumor-initiating systems we used.

Inactivating mutational events could also have occurred within the translated region of the gene but outside our scope of evaluation. In the present study, we focused on our analysis on an 817-base pair fragment of the rat p53 gene containing the conserved coding regions of exons 4–10. The rat p53 gene sequence is at least 12 kilobases long (42) and contains an as yet undefined length of upstream translational regulatory sequences in exon 1 (51). The transcriptional regulatory region of the the rat p53, containing an unknown array of repressor, enhancer, and promoter elements, is equally undefined. Additionally, at least one essential posttranscriptional processing regulatory element is known to exist within the fourth intron (52). Potential mutations located within these regulatory elements could render the gene inactive but would not have been detected in this study. Similarly, potential mutations affecting mRNA splicing within introns or at intron/exon splice sites (53) may potentially be missed in the present study since the 817-base pair PCR fragment was reverse transcribed from expressed mRNA. It should be noted that there was no indication of splice-variants of the 817-base pair fragment with the cDNA primers used.

When reporting negative results such as these, it is important to give some perspective as to levels of sensitivity and confidence. We have included positive controls in our analysis which allow us to gauge the level at which dilution by normal cells or nonmutated tumor cells (if p53 mutation is a late arising event) would mask the presence of mutations. We estimate that in our hands CCM will detect most mutations at a level of 1 in 20 gene copies, that SSCP will detect at levels of 1 in 10, and direct sequencing will detect levels of 1 in 8 (54). While the SSCP technique is more sensitive than direct sequencing in our hands, the inherent faults of SSCP analysis include the reality that not all point mutations will be identified, especially if the migration rates of the wild type and certain mutant single-stranded conformers are similar. In contrast, the CCM technique is very sensitive and reliable. Its heightened sensitivity compared to direct sequencing makes CCM more useful for surveying large DNA frag-
ments for unknown point mutations. Utilization of techniques which are orders of magnitude more sensitive, such as allele-specific PCR, oligonucleotide hybridization, or RFLP, is not possible when scanning for random unknown mutations. Given that the tumors were harvested late in tumor development, ensuring that genes which have critical roles should be expressed in most cells, we are confident that point mutations within the coding region, with one exception, are not present in the majority of RMT cells in the regions of the p53 gene we analyzed.

REFERENCES


Low Incidence of Point Mutations Detected in the p53 Tumor Suppressor Gene from Chemically Induced Rat Renal Mesenchymal Tumors

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