Selective Mutation of Codons 204 and 213 of the p53 Gene in Rat Tumors Induced by Alkylating N-Nitroso Compounds1

Hiroko Ohgaki, Gordon C. Hard, Norio Hirota, Akihiko Maekawa, Michihito Takahashi, and Paul Kleihues2

Institute of Neuropathology, University of Zurich, CH-8091 Zurich, Switzerland [H. O., P. K.]; American Health Foundation, Valhalla, New York 10595 [G. C. H.]; Department of Pathology, Jichi Medical School, Kawachi-gun, Tochigi 329-04 Japan [N. H.]; Department of Pathology, Sasaki Institute, Chiyoda-ku, Tokyo 101, Japan [A. M.]; and Division of Pathology, National Institute of Health Sciences, Setagaya-ku, Tokyo 158, Japan [M. T.]

Abstract

Kidney and esophageal tumors induced by alkylating N-nitroso compounds in rats contain a high incidence (75-100%) of G  →  T transition mutations in the p53 gene. These are almost selectively (89%) located in the first base of codon 204 and the second base of 213, leading to amino acid substitutions Glu  →  Lys and Arg  →  Gln, respectively. In contrast to human neoplasms, a considerable fraction of rat kidney and esophageal tumors carries multiple p53 mutations. All nephroblastomas induced by transplacental exposure to N-nitrosoethyurea and 56% of esophageal tumors induced by N-nitrosomethyurea showed double mutations in codons 204 and 213 of exon 6. The selective targeting of p53 codons by alkylating nitrosamines may provide a basis for molecular epidemiological studies on this class of chemical carcinogens.

Introduction

The pattern of point mutations in the p53 tumor suppressor gene varies considerably in human neoplasms and appears to be related to organ site. G:C to A:T transitions predominate in colon, brain, and lymphoid malignancies, whereas G:C to T:A transversions are most frequent in lung and liver cancers (1). In addition, specific etiological agents may produce typical mutational hotspots. This was first shown for hepatocellular carcinomas from high-incidence areas in South Africa and China, which in 30-40% contain G → T transversion in codon 249 (2, 3). This mutation is assumed to result from the interaction of aflatoxin B1 with cellular DNA and is not observed in hepatocellular carcinomas from patients with hepatitis B virus infection (4). UV light typically induces pyrimidine dimers in skin DNA, and a recent report by Brash et al. (5) showed that squamous cell carcinomas of the skin contain in more than one-half of cases CC to TT mutations, i.e., double-base changes that are likely to be due to miscoding by UV light-induced thymine dimers. N-nitroso compounds constitute a group of powerful chemical carcinogens which in experimental animals induce a high incidence of tumors with a remarkable degree of organ specificity (6). Although N-nitroso compounds have been implicated as contributors to the overall human tumor load, it has proven difficult to identify human neoplasms specifically induced by nitrosamines (7). We have, therefore, analyzed a variety of tumors induced by N-nitroso compounds in rats and found a high incidence of p53 mutation, clustered in two codons of exon 6.

Received 2/20/92; accepted 4/14/92.

1 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
2 To whom requests for reprints should be addressed, at Department Pathology, Universität Zurich, Schmelzbergstrasse 12, 8091 Zurich, Switzerland.

Materials and Methods

Induction of Tumors. Renal mesenchymal tumors were induced in Charles River Wistar rats treated at 5 weeks of age with a single i.p. 40 mg/kg dose of NDMA1 (8). Carcinomas (epithelial tumors originating from renal tubules) were induced by the same treatment regimen, except that the dose was 30 mg NDMA/kg, and the age of the rats at treatment was 9-10 weeks (8). Nephroblastomas were induced in Nb hooded rats by a single i.p. (transplacental) exposure to NEU on day 18 of gestation at a dose of 60 mg/kg body weight (9). Spontaneous nephroblastomas from a breeding colony of inbred WAB/Not rats were also examined. These tumors occurred in 0.5% of breeders surviving up to 2 years of age, representing 40% of the total spontaneous tumors in this rat strain (10). Esophageal papillomas and carcinomas were induced in F344 rats by exposure to NMU in the drinking water (200 ppm) for 42 weeks (11). Adenocarcinomas in the glandular stomach were induced in F344 rats by NMU in the drinking water (200 ppm; 15 weeks) (12), by intragastric (gavage) doses of NMU (4 mg/kg each), or in newborn Brown Norway rats by a single dose of NMU (200 mg/kg body weight). Oligodendrogliomas were induced in fetal brain transplants by two doses of NEU (50 mg/kg each), using adult F344 rats as recipients (13).

Single-Strand Conformation Polymorphism Analysis. DNA was extracted from paraffin sections by the procedure described by Shibata et al. (14) with slight modifications as follows. After comparison with serial sections stained with hematoxylin and eosin, tumors were scraped off the histological slide. Samples were deparaffinized with xylene and washed with absolute ethanol. Dried samples were treated with 500 μg/ml of protease K (Boehringer Mannheim GmbH, Mannheim, Germany) in 40-80 μl of digestion buffer (50 mM Tris, pH 8.5; 1 mM EDTA; and 0.5% Tween 20) at 55°C for 3 h. After inactivation of protease K by heating at 95°C for 10 min, samples were kept at -20°C until PCR reaction.

Pairs of 20-mer oligonucleotide primers corresponding to exons 5-8 of p53 gene were synthesized on a model 391 PCR-MATE DNA synthesizer (Applied Biosystems, Inc.). The sequences of PCR primers were 5'-ACTCAATTTCCTTCAATAAG and 5'-CCATCGAG-GAGCTCA for exon 5, 5'-GGCTGGCTCTCCCACA and 5'-GGTGGCTCATACGGGACG for exon 6, 5'-GTCGGCTCCG-...
The reaction mixture (1.5 μl) was mixed with 2 μl of 0.1 M NaOH and 9 μl of sequencing stop solution (USB). Samples were heated at 95°C for 10 min and immediately loaded onto a 6% polyacrylamide nondenaturing gel containing 7.5% glycerol. Gels were run at 7 W for 13–15 h at room temperature. Gels were dried at 80°C, autoradiographed with an intensifying screen for 5–48 h, and the pattern of single-stranded DNA was checked for differences.

Direct DNA Sequencing of PCR Products. For exon 6, all samples were analyzed by direct sequencing of PCR products. For exons 5, 7, and 8, direct sequencing was only carried out on samples which had scored positive in single-stranded conformation polymorphism analysis. PCR was performed in a total volume of 80 μl, as described above. After amplification, 70 μl of the PCR reaction mixture were separated on a 6% polyacrylamide gel. Amplified bands were cut out; eluted in 0.5 M ammonium acetate, and 1 mM EDTA at 37°C overnight, and precipitated with ethanol. Dried DNA was resuspended in 15 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Sanger dideoxynucleotide sequencing was performed using [α-32P]dCTP and amplification primers. The template-primer mixture (4 μl DNA and 10 pmol primer) was heated at 95°C for 5 min and immediately placed in liquid nitrogen. An aliquot containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 25 mM NaCl, 0.1 mM dithiothreitol, 10% dimethyl sulfoxide, and 5 μCi [α-32P]dCTP was added to each of the four termination mixtures and incubated at 37°C for 10 min with 2 units of Sequenase, version 2.0 (USB). Samples were mixed with 4 μl stop solution, heated at 80°C for 2 min, and immediately loaded onto a 6% polyacrylamide/7 M urea gel. Gels were fixed with 10% acetic acid and 10% methanol, dried, and autoradiographed for 5 h to 3 days. When mutations were detected, they were further confirmed by sequencing the opposite strand. In each reaction, normal rat kidney DNA was concurrently amplified and sequenced as a negative control.

Results

Independent of the histopathologic tumor type and mode of induction, all rat kidney tumors investigated contained a high incidence (75–100%) of p53 mutations (Table 1). These were heavily clustered in codons 204 and 213 of exon 6. Often, both exons were mutated in the same neoplasm. This was most prominent in nephroblastomas induced by transplacental exposure to NEU, all of which contained G → A transitions in codons 204 and 213. Often, both exons were mutated in the same neoplasm. This was most prominent in nephroblastomas induced by transplacental exposure to NEU, all of which contained G → A transitions in codons 204 and 213. Often, both exons were mutated in the same neoplasm. This was most prominent in nephroblastomas induced by transplacental exposure to NEU, all of which contained G → A transitions in codons 204 and 213.

Table 1

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Carcinogen</th>
<th>Exon 5</th>
<th>Exon 6</th>
<th>Exon 7</th>
<th>Exon 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinoma</td>
<td>NDMA</td>
<td>7/8 (88%)</td>
<td>1*</td>
<td>7 (88%)</td>
<td>0</td>
</tr>
<tr>
<td>Mesenchymal tumor</td>
<td>NDMA</td>
<td>6/8 (75%)</td>
<td>1*</td>
<td>4 (50%)</td>
<td>0</td>
</tr>
<tr>
<td>Nephroblastoma</td>
<td>NEU</td>
<td>8/8 (100%)</td>
<td>0</td>
<td>8 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Nephroblastoma</td>
<td>Spontaneous</td>
<td>8/12 (75%)</td>
<td>0</td>
<td>9 (75%)</td>
<td>0</td>
</tr>
<tr>
<td>Esophagus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinoma/papilloma</td>
<td>NMU</td>
<td>7/9 (78%)</td>
<td>0</td>
<td>6 (67%)</td>
<td>0</td>
</tr>
<tr>
<td>Glandular stomach</td>
<td>NMU</td>
<td>1/2 (8%)</td>
<td>1*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligodendroblastoma</td>
<td>NEU</td>
<td>0/7 (0%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Codon 140, ACA → ACG; Thr → Thr.
* Codon 135, TGC → TAC; Cys → Tyr.
* Codon 274, GTT → ATT; Val → Ile.
* Codon 238, TGC → TGT; Cys → Cys.
* Codon 238, TGC → TAC; Cys → Tyr.
* Codon 243, TGC → TAC; Cys → Tyr, and codon 239, AAC → AAT; Asn → Asn.
* Codon 144, CAG → CAA; Glu → Gln.

Discussion

DNA sequence analyses were carried out on a variety of tumors induced in rats by methylyating or ethylating N-nitroso compounds, using formalin-fixed, paraffin-embedded tissue samples (Fig. 1). We found a high incidence of miscoding p53 mutations (75–100%) in tumors of the kidney and esophagus but not in neoplasms induced by the same carcinogens in stomach and brain. This suggests that the involvement of p53 inactivation during carcinogenesis occurs with a high degree of tissue specificity. It has previously been shown that NEU-induced gliomas of the central nervous system do not carry ras mutations (16), and when we analyzed NMU-induced glandular stomach carcinomas, we were also unable to detect mutations in the H- and K-ras genes. In contrast, tumors which in the present study were found to carry p53 mutations are also known to have a high incidence of K-ras (kidney) and H-ras (esophagus) mutations (17, 18). It thus appears that in some tissues these transformation-associated genes cooperate in malignant transformation (19). The simultaneous occurrence of p53 and ras mutations has also been observed in human colorectal cancer (20, 21) but does not seem to apply to all tissues. In studies on human esophageal carcinomas from high-incidence areas, p53 base substitutions were found in 44% of neoplasms, while ras mutations were completely absent (22).

Regarding the location of p53 mutations, the results obtained were unexpected in two respects. Rather than being randomly...
distributed over exons 5 to 8, mutations were heavily (89%) clustered in exon 6, and within this exon they selectively affected codons 204 and 213, leading to amino acid substitutions Glu → Lys and Arg → Gin, respectively (Table 1). Point mutations in codon 204 have not previously been reported in any human or animal neoplasm. Codon 213, on the other hand, is a fairly frequent site for p53 substitution mutations in a variety of human neoplasms (1, 23). Furthermore, the third base of codon 213 shows an approximately 2–11% background level of CGA → CGG (Arg → Arg) polymorphism (24–26). In the rat, this mutation creates a typical intron-exon splicing acceptor sequence. Another unexpected finding was the simultaneous presence in renal (42%) and esophageal tumors (56%) of mutations in both hotspot codons. This was particularly evident in nephroblastomas transplacentally induced in rats by NEU (9). All eight tumors analyzed contained mutations in both codons 204 and 213, in addition to a G → A transition mutation in codon 12 of the K-ras gene (17). It remains to be clarified whether these double mutations are present in the same tumor cell population or whether there are different neoplastic cell types containing point mutations at either codon 204 or 213. In human neoplasms, double mutations appear to be a very rare event (26, 27).

It has been established that O-alkylated bases are the major promutagenic DNA lesions produced by simple alkylating N-nitroso compounds. The pathway for production of point mutations by O-k-methyl- and O-k-ethyldeoxyguanosine is mispairing during DNA replication with deoxycytidine (28). Our finding that most base substitutions are G → A transitions suggests that O-k-alkyldeoxyguanosines are responsible for these mutations.

There is increasing evidence that chemical carcinogens or their ultimate reactive metabolites cause specific base substitutions in oncogenes and tumor suppressor genes (2, 3, 29–31). Our observation of the same mutational hotspots in tumors of two different tissues (kidney and esophagus) and in three histologically different types of kidney tumors (carcinomas, mesenchymal tumors, and nephroblastomas) suggests a selective targeting of codons 204 and 213 in neoplasms induced by alkylating N-nitroso compounds in rats. Identification of such selective mutations in human neoplasms would allow an estimation of the actual contribution of carcinogenic N-nitroso compounds to the overall human tumor load. However, this is the first report on p53 mutations in rats, and it cannot be excluded that mutations at these sites predominate because they lead to a species-specific growth advantage of initiated cells, irrespective of the chemical nature of the inducing agent. Also, site-specific DNA repair deficiency may contribute to the selective mutations of codons 204 and 213 (28). To resolve this question, analyses of a wide range of chemically induced animal tumors will be necessary. However, it may be worthwhile to analyse exon 6 of p53 in WBC (32) and in second primary malignancies of patients who underwent chemotherapy with methylating (procarbazine, dacarbazine, streptozotocin) or chloroethylnitrosourea [1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea] agents.

It is noteworthy that spontaneous nephroblastomas in WAB/Not rats showed p53 point mutations similar to, but less frequently than, nephroblastomas induced by NEU (Table 1). The pathogenesis of spontaneous nephroblastomas has not been fully elucidated (10). Since their incidence is very low (0.5% of breeders surviving up to 2 years), it is unlikely that these neoplasms are due to oncogenic viruses or genetic predisposition. Our findings suggest rather that nephroblastomas in WAB/Not rats are not truly spontaneous but may result from low-level dietary exposure to environmental N-nitroso compounds or related carcinogens.

It has been suggested that the biological properties of mutant p53 proteins depend on the site of point mutations. Base substitutions at codons 151, 247, and 273 exert a dominant negative effect on the phenotype of cotranslated wild-type human p53, driving the latter into the mutant phenotype form, whereas the p53 protein encoded by a mutated codon 248, i.e., the germ-line mutation underlying the Li-Fraumeni syndrome (33, 34), does not show this effect (35). Studies are under way to determine the effects of point mutations at codons 204 and 213 of the rat p53 gene and their possible relationship to organ-specific malignant transformation.

Human tumors frequently exhibit either a loss of both alleles of the p53 gene, the loss of one p53 allele with an associated point mutation, insertion or deletion of the remaining allele, or an inactivation of the p53 gene in one allele but a normal (wild-type) sequence in the other. In the present study, sequencing gels showed consistently weaker signals for the mutated base when compared to the corresponding normal (wild-type) sequence (Fig. 1). This may be due to the fact that only a fraction of tumor cells carry the mutation. A loss of the normal allele was observed in only one renal mesenchymal tumor (Fig. 1), but Southern blot analyses with highly polymorphic markers may reveal a higher incidence of allelic loss.

In conclusion, this study shows that renal and esophageal tumors induced by carcinogenic N-nitroso compounds in rats...
contain an unusually high incidence of p53 mutations which are clustered in codons 204 and 213. Unlike human neoplasms, a high proportion of tumors contain double mutations in both hotspot codons.

References


Selective Mutation of Codons 204 and 213 of the p53 Gene in Rat Tumors Induced by Alkylating N-Nitroso Compounds


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/52/10/2995

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.