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Rat p53 gene mutations in primary Zymbal gland tumors induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline, a food mutagen

(guanine → pyrimidine transversion/deletion mutation/mutation specificity/mRNA from the wild-type allele)

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ABSTRACT **There are reports of p53 gene mutations in various human cancers but not in rat tumor cell lines or rat primary tumor tissue. We found a p53 gene mutation in a cell line of a spontaneous squamous cell carcinoma of the rat Zymbal gland, SCC131, at codon 171 by direct sequencing of cDNA fragments amplified by PCR. We tested for p53 gene mutations in 15 primary Zymbal gland tumors induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline by single-strand conformation polymorphism analysis of the PCR-amplified cDNA products. Samples of four tumors showed mobility shifts. Direct sequencing revealed that all these tumors had mutations in conserved regions or in scattered conserved residues. Single-strand conformation polymorphism analysis of cDNA suggested that mRNA from the wild-type allele of the p53 gene was not present in tumor cells of three of four positive cases, although genomic DNA analysis indicated that the wild-type allele was retained in all the cases. All mutations were found at a guanine base: three mutations were guanine → pyrimidine transversions and one was a deletion of a guanine base within a G+C-rich sequence. These findings indicate that 2-amino-3-methylimidazo[4,5-*f*]quinoline may be directly involved in induction of these mutations by forming DNA adducts at various sites in the p53 gene.**

Various human tumors have been demonstrated to be associated with loss of heterozygosity of a 17p chromosome fragment bearing the p53 gene (1, 2). These tumors include colon, breast, liver, lung, stomach, esophagus, bladder, and bone tumors (3–10). In most cases, the remaining p53 genes in these tumors had single-base substitutions in the regions that are conserved among various animal species (11), and the normal p53 protein was not produced.

The wild-type p53 gene encodes a 53-kDa nuclear protein that functions as a tumor suppressor (12). Some of the mutated p53 genes were found to have acquired transforming activity, suggesting that the mutated p53 protein functions in a dominant-negative fashion (13).

Recently, p53 mutations in a series of cell lines derived from methylcholanthrene-induced mouse fibrosarcomas have been reported (14). To our knowledge, there have been no reports, however, on the involvement of the p53 gene in rat carcinogenesis. Various carcinogens have been shown to induce specific mutations. For instance, *N*-nitroso-*N*-methylurea specifically induces a G → A mutation at the second position ("letter") of codon 12 in the Ha-*ras* gene of various organs of different animals (15–17). These findings prompted us to analyze the effect of a food carcinogen, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), on the p53 gene in rats to determine whether the p53 gene is involved in rat carcinogenesis and, if so, to verify whether IQ induces position-specific mutations in the p53 gene, the mutation sites of which are variable in human tumors.

IQ is present in cooked meat and fish (18–21) and cigarette smoke (22). It is a very strong mutagen in *Salmonella typhimurium* (23) and also induces mutations in Chinese hamster lung cells (24). It induces liver and lung tumors in CDF_1 mice (25) and liver and Zymbal gland tumors in F344 rats (26). Recently, IQ was also proved to be carcinogenic in a nonhuman primate; it induced liver cancer (27). Exposure to IQ is a risk factor for human carcinogenesis, so somatic genetic changes induced by this compound are of great interest.

In this study we used single-strand conformation polymorphism (SSCP) analysis (28, 29), which is a convenient and rapid method for screening large numbers of tumors. First we analyzed the p53 cDNA in two rat cell lines by direct sequencing of PCR-amplified products and found a mutation in a cell line of a spontaneous squamous cell carcinoma of the rat Zymbal gland. Using this cell line as a positive control, we screened for p53 gene mutations by SSCP analysis of PCR-amplified cDNA (hereafter referred to as cDNA-PCR-SSCP analysis) in 15 primary Zymbal gland tumors of rats induced by IQ in a region that included four hot spots (A–D) (3) and four conserved regions (II–V) (11).

MATERIALS AND METHODS

Cell Lines. Cells of L6TG, a rat myeloblast cell line, were cultured in Eagle's minimum essential medium with 10% fetal calf serum. SCC131 cells, a cell line of a spontaneous squamous cell carcinoma of the rat Zymbal gland, were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. L6TG and SCC131 were obtained from the Japanese Cancer Research Resources Bank.

Primary Rat Zymbal Gland Tumors. Six-week-old male F344 rats (Charles River Japan) were given 0.03% IQ in their diet. Zymbal gland tumors were induced in 25 of 26 rats examined during the 49-week experimental period. Of these, 15 tumors from 14 rats were examined for p53 mutations. Histologies were examined after hematoxylin/eosin staining.

Preparation of cDNA. Total cellular RNAs from cell lines and primary tumors were prepared by the acid guanidinium thiocyanate/phenol/chloroform extraction method (30). Contaminating DNA in samples of ≈50 μg of RNA was digested with 2 units of RNase-free DNase I (RQ1; Promega) in the presence of RNasin (Toyobo, Osaka) at 37°C for 30 min, and RNA was purified by phenol/chloroform extraction and ethanol precipitation (31). Samples of 1 μg of RNA were

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Abbreviations: IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; SSCP, single-strand conformation polymorphism; nt, nucleotide(s).

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denatured at 68°C for 10 min and annealed with 100 pmol of random hexamers (Pharmacia), and cDNA was synthesized in a 20-μl reaction mixture with an avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) at 42°C for 60 min (32).

Preparation of DNA. DNAs from primary tumors were prepared according to a reported method (33). Briefly, a small piece of tissue was minced in 1 ml of PCR buffer supplemented with 0.45% Nonidet P-40 (Sigma), 0.45% Tween 20 (Bio-Rad), and proteinase K at 10 mg/ml; incubated at 55°C for 1 h; and then incubated at 90°C for 10 min to inactivate proteinase K. The mixture was then centrifuged.

Direct DNA Sequencing. Aliquots (2 μl) of cDNA from the 20-μl reaction mixture described above were amplified by an asymmetric PCR with 2.5 units of *Taq* DNA polymerase (Takara Shuzo, Kyoto) by using 50 and 5 pmol of paired primers (34). The design and sequences of the primers are depicted in Fig. 1 and Table 1. Primers were synthesized by the phosphoroamidite method in a 381A DNA synthesizer and purified on an oligonucleotide purification cartridge (Applied Biosystems). Fifty cycles of the reaction at 94°C, 55°C, and 72°C for 0.5, 1, and 2 min, respectively, were carried out in a Perkin–Elmer/Cetus thermal cycler. After phenol/chloroform extraction and ethanol precipitation, unincorporated nucleotides and primers were removed from amplified DNAs by filtration through a polysulfone filter with a molecular weight cutoff of 10,000 (Ultrafree C3GC; Millipore). The primer for sequencing (see Fig. 1 and Table 1) was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase

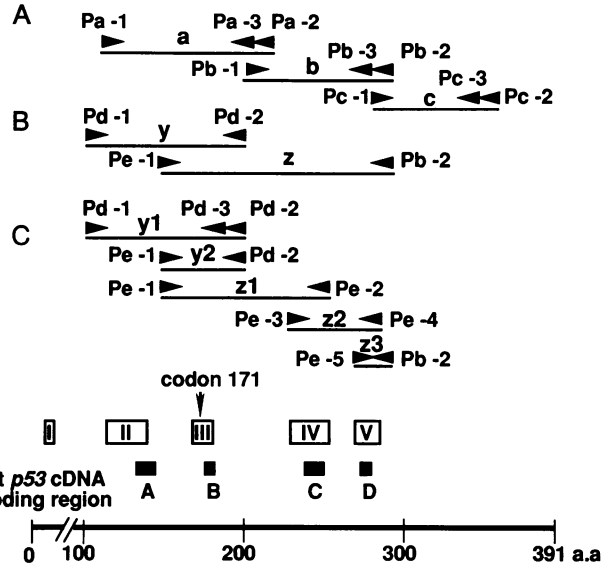

FIG. 1. Design for analysis of rat p53 mutations in cDNA. Solid boxes show four hot spots (A–D) reported for human tumors (3), and open boxes (I–V) show the five conserved regions (11). (*A*) Design for direct sequencing of PCR products. A 674-base-pair fragment [nucleotides (nt) 366–1040] that includes four hot spots was divided into three fragments (a, b, and c). For analysis of two cell lines (SCC131 and L6TG), primers were designed for asymmetric PCR at both sides of three fragments (Pa-1 and Pa-2; Pb-1 and Pb-2; Pc-1 and Pc-2) and for sequencing (Pa-3, Pb-3, and Pc-3). (*B*) Design for cDNA PCR-SSCP analysis. The fragment including conserved regions II–V and four hot spots was divided into two fragments (y and z), and primers were designed so that these two fragments included codon 171 (Pd-1 and Pd-2; Pe-1 and Pb-2). Fragment y was from nt 307 to nt 603, and fragment z was from nt 445 to nt 881. (*C*) Sequencing strategy for fragments y and z. Primer designs for analysis of the y fragment (y1 and y2) and the z fragment (z1, z2, and z3) are shown. For asymmetric PCR amplification of the five fragments, eight primers (Pd-1, Pd-2, Pe-1, Pe-2, Pe-3, Pe-4, Pe-5, and Pb-2) were used. For sequencing, five primers (Pd-3, Pd-2, Pe-2, Pe-4, and Pb-2) were used. a.a., Amino acid.

Table 1. Oligonucleotide primers

Name	Sequence
Pa-1	5′-ACGTACTCAATTTCCCTCAA-3′
Pa-2	5′-CTCAGGTGGCTCATACGGTA-3′
Pa-3	5′-CACCACGCTGTGCCGAAAAG-3′
Pb-1	5′-GAGTATCTGGACGACAGGCA-3′
Pb-2	5′-CAATGCTCTTCTTTTTTGCG-3′
Pb-3	5′-TTCTTTTTTGCGGAA-3′
Pc-1	5′-CGTCGGACAGAGGAAGAAAA-3′
Pc-2	5′-TCCAAGGCCTCATTCAGCTC-3′
Pc-3	5′-GAACATCTCGAAGCGCTCAG-3′
Pd-1	5′-GGCAACTATGGCTTCCACCT-3′
Pd-2	5′-AGCATACGGATTTCCTTCCA-3′
Pd-3	5′-CCAGGTGGAGGTGTGGA-3′
Pe-1	5′-CCTCCACCTGGTACCCGTGT-3′
Pe-2	5′-GAGTCTTCCAGCGTGATGAT-3′
Pe-3	5′-TATACCACTATCCACTACAAG-3′
Pe-4	5′-ATTTTCTTCCTCTGTCCGAC-3′
Pe-5	5′-GGGAATCTTCTGGGACGGGA-3′
P5-1	5′-GATTCTTTCTCCTCTCCTAC-3′
P5-2	5′-ACAGGCAGTGCCAGTGCTCA-3′
P6-1	5′-CCCGGCCTCTGACTTATTCT-3′
P6-2	5′-CTCAGGTGGCTCATACGGTA-3′
P7-1	5′-TATACCACTATCCACTACAAG-3′
P7-2	5′-CCCAACCTGGCACACAGCTT-3′

(28), and the sequencing reaction was performed with Sequenase version 2.0 (United States Biochemical) according to the manufacturer's instructions.

Cloning of PCR Products and DNA Sequencing. After phenol/chloroform extraction and ethanol precipitation, the amplified DNA was treated with T4 polymerase and ligated to the *Eco*RV site of pBluescript SK(+) (Stratagene). Plasmids with inserts were selected by picking up white colonies in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactoside and isopropyl β-D-thiogalactoside. Sequencing was performed on alkali-denatured plasmids.

PCR-SSCP Analysis. PCR-SSCP analysis was performed by a reported method (28, 29). A 1-μl sample of the 20-μl cDNA reaction mixture or 1 μg of DNA was amplified by PCR with 0.7 unit of *Taq* polymerase and 15 pmol of two end-labeled oligonucleotide primers (35). Primers were designed to generate fragments y and z of the p53 cDNA and fragments of exons 5, 6, and 7 of the p53 gene (Fig. 1*B*, Fig. 2, and Table 1). Thirty cycles of the reaction were performed at 94°C, 55°C, and 72°C for 0.5, 0.5, and 1 min, respectively. Then 1 μl of the 25-μl PCR reaction mixture was mixed with 99 μl of 0.1% SDS/10 mM EDTA, and 2 μl of this solution was mixed with 2 μl of 95% formamide/20 mM EDTA/0.05% bromophenol blue/0.05% xylene cyanol, heated at 80°C for 2 min, and applied (1 μl per lane) to a 6% polyacrylamide gel containing 90 mM Tris borate (pH 8.3) and 4 mM EDTA. Electrophoresis was performed at 40 W for 2–2.5 h. The gel was dried on filter paper and exposed to an imaging plate

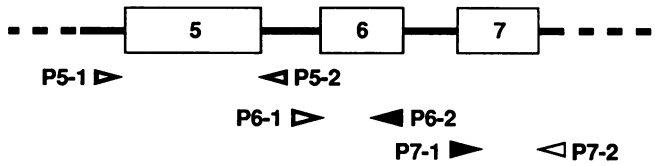

FIG. 2. Design for analysis of rat p53 mutations in genomic DNA. Open boxes show exons. Open arrowheads show primers from intron sequences, and filled arrowheads show primers from exon sequences. Primers were designed to analyze exons 5, 6, and 7 where p53 mutations of four tumors (tumors 3, 5, 19, and 21) found in cDNA are located. Six primers (P5-1, P5-2, P6-1, P6-2, P7-1, and P7-2) were used for PCR-SSCP analysis.

(Fuji), and signals were analyzed with an image analyzer (Fuji).

RESULTS

Detection of p53 Mutations in cDNA of SCC131 by Direct Sequencing. The nucleotide sequences of the p53 cDNAs from SCC131, a Zymbal gland tumor cell line, and L6TG, a myeloblast cell line, were first determined by the direct sequencing method. The region from nt 366 to nt 1040 including the four hot spots for p53 mutations of human tumors (3) was divided into three overlapping fragments: a, b, and c (Fig. 1*A*), and each fragment was amplified by asymmetric PCR. The first letter of the initiation codon was designated nt 1. A change of G → A was found at the first letter of codon 171 in p53 cDNA of SCC131 (data not shown) by comparison with the reported rat p53 cDNA sequence (36). This mutation resulted in substitution of methionine for valine. The nucleotide sequencing data suggested that mRNA from the wild-type allele of the p53 gene was not present because only A, but no G, was detected as the first letter of the codon. No mutations were detected in fragment a, b, or c of L6TG.

Detection of p53 Mutations in cDNA of Primary Zymbal Gland Tumors by SSCP. We used cDNA-PCR-SSCP analysis with SCC131 and L6TG cDNAs as positive and negative controls, respectively, for screening p53 gene mutations of tumors. The region from nt 307 to nt 881 of the coding region, which includes the four conserved regions (II–V) (11), was divided into two overlapping regions, y and z, both of which include codon 171 where p53 of SCC131 was mutated (Fig. 1*B*). Upon electrophoresis at room temperature without glycerol, SCC131 showed mobility shifts of both the y and z bands from those of L6TG (Fig. 3*A*, lanes 5 and 6). It was clearly shown that mRNA from the wild-type allele was not present in SCC131, in accordance with the results of direct sequencing. Furthermore, PCR-amplified DNA bands of exon 5 of SCC131, in which the mutated codon is located, showed mobility shifts from those of normal rat liver DNA (Fig. 3*C*). The wild-type allele was not detected.

The p53 mutations in 15 original Zymbal gland tumors induced by IQ were analyzed under the same conditions by using PCR-amplified cDNA. Both strands of tumor 5 and the faster moving strand of tumor 19 showed mobility shifts of both the y and z bands (Fig. 3*A*, lanes 2 and 3). The faster moving strand of tumor 3 and the slower moving strand of tumor 21 showed mobility shifts of the z band only (Fig. 3*A*, lanes 1 and 4). The intensities of the shifted bands, if present, in all the SSCP-positive tumors except tumor 19 were stronger than those of bands of the wild-type allele transcript. Since the amounts of the mutated and wild-type PCR-amplified cDNA products should be well correlated with the amounts of mRNAs in the starting material (31), mRNAs from the wild-type allele were considered to be present in reduced amounts, if at all, in these tumors. Some or all of the wild-type transcripts detected should be derived from stromal cells present with the tumor cells. The y and z bands of the other 11 tumors showed the same mobilities as those of L6TG, a negative control (data not shown).

Determination of the p53 Mutations in cDNAs of the Primary Tumors. Tumors 5 and 19 showed mobility shifts of both the y and z fragments, indicating that mutations were probably in only the y2 fragment, the region where the y fragment overlapped the z fragment (see Fig. 1). First the y2 fragments of tumors 5 and 19 were amplified with Pe-1 and Pd-2 (10:1, mol/mol) as primers and then directly sequenced with Pd-2 as a sequencing primer. In tumor 5, a G → T transversion was found at the second letter of codon 174, resulting in a cysteine → phenylalanine substitution (Fig. 4). Only a faint band of the guanine signal was detected at the mutated site in the sequence gel, suggesting the presence of little if any mRNA from the wild-type allele in the tumor cells, in accordance with SSCP analysis (Fig. 3*A*, lane 2). Direct sequencing of the y2 fragment of tumor 19 indicated a C → G transversion at the first letter of codon 156, resulting in a glycine → arginine substitution (data not shown). In this case, the cytidine signal of the wild-type allele was stronger at the mutation site (data not shown), which is consistent with the results obtained by SSCP. Fragments y1, z1, z2, and z3 of tumors 5 and 19 were analyzed by direct sequencing, but no other mutations were detected in any of these fragments from either tumor.

Tumors 3 and 21 showed mobility shifts of only the z region. The z fragment was divided into three parts, z1, z2, and z3, to determine the mutation sites by direct sequencing

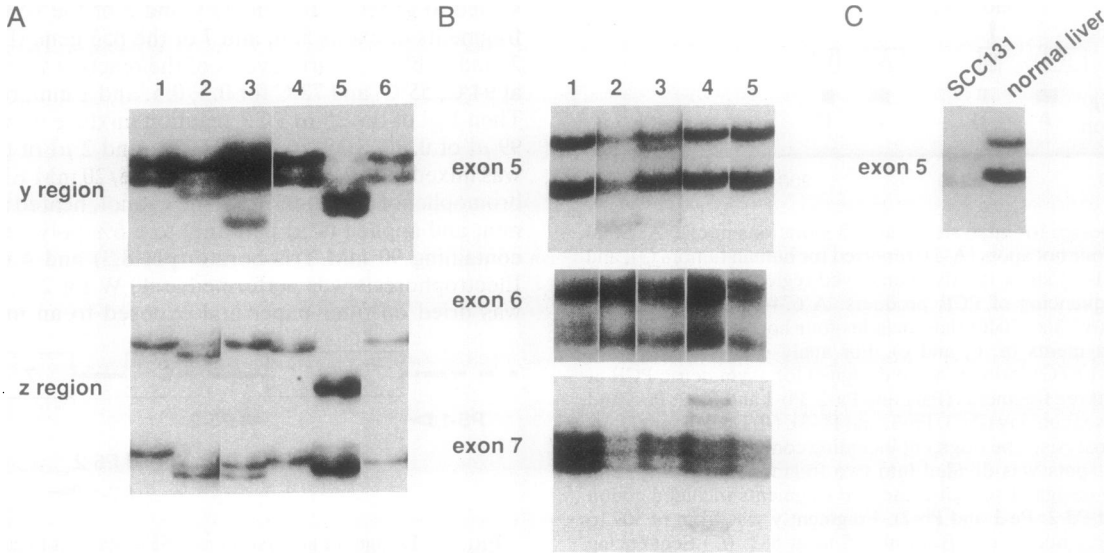

FIG. 3. Detection of p53 mutations in SCC131 and IQ-induced Zymbal gland tumors by PCR-SSCP analysis. (*A*) cDNA-PCR-SSCP analysis of the y and z fragments. Lanes 1–4 contain samples from tumors 3, 5, 19, and 21, respectively. Lanes 5 and 6 contain samples from cell lines SCC131 and L6TG, respectively. (*B*) PCR-SSCP analysis of exons 5, 6, and 7 in IQ-induced Zymbal gland tumors. Lanes 1–4 contain samples from tumors 3, 5, 19, and 21, respectively. Lane 5 contains a DNA sample from normal rat liver. (*C*) PCR-SSCP analysis of exon 5 in cell line SCC131.

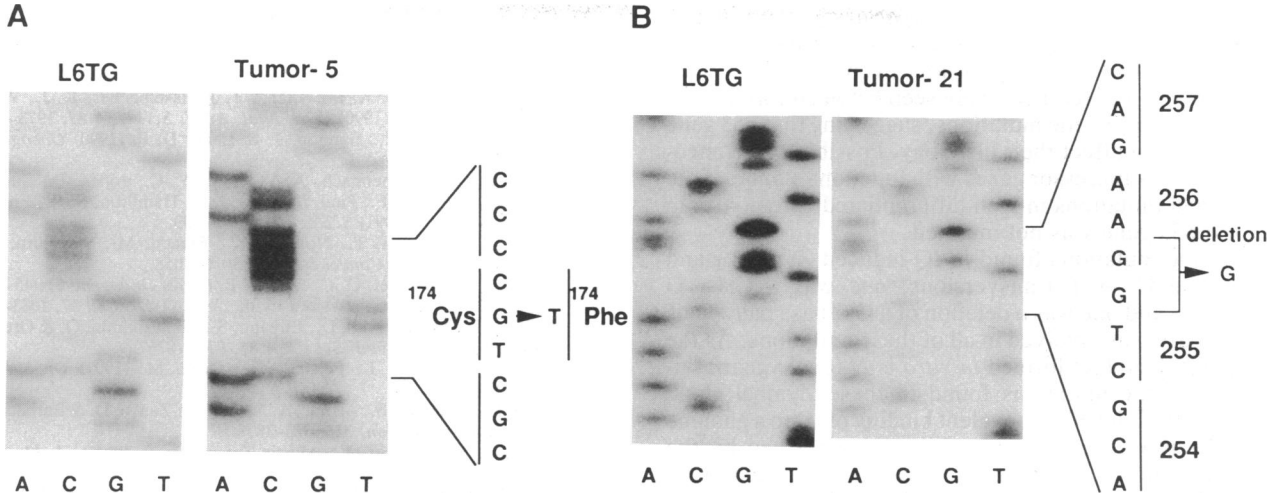

FIG. 4. Nucleotide sequences of mutated p53 in IQ-induced Zymbal gland tumors. (*A*) Direct sequencing of the y2 fragment of tumor 5. A G → T transversion at the second letter of codon 174 is observed. (*B*) Nucleotide sequencing of a subclone of the z2 fragment of tumor 21. The third position of codon 255 or the first position of codon 256 is deleted.

(Fig. 1). In tumor 3, a G → T transversion was found at the first letter of codon 214, resulting in a valine → leucine substitution in the z1 fragment. Tumor 21 had a G·C base pair deletion at the third position of codon 255 or the first position of codon 256 in the z2 fragment. This deletion was confirmed by sequencing the cloned PCR products: three of five subclones showed the deletion (Fig. 4*B*). The deletion caused a frameshift and the appearance of a termination codon at codon 347, resulting in changes of amino acids starting from codon 256. The findings that the wild-type guanine signal at the mutated site of tumor 3 and those downstream to the deletion site of tumor 21 were faint on direct sequencing analysis suggested that little, if any, mRNA from the wild-type allele was present in cells of either tumor.

By histological examination of the Zymbal gland tumors, 13 tumors were diagnosed as well-differentiated squamous cell carcinomas and 2 tumors were diagnosed as papillomas (data not shown). The four tumors with p53 gene mutations, tumors 3, 5, 19, and 21, were all well-differentiated squamous cell carcinomas.

Detection of p53 Gene Mutations in Primary Zymbal Gland Tumors by SSCP. Since the mutations found in cDNA samples of tumors were located in exon 5 (codons 156 and 174), exon 6 (codon 214), and exon 7 (codon 256), SSCP analysis was performed on DNA fragments of these exons from the tumors with or without addition of 5% glycerol at room temperature. Tumors 5 and 19 showed mobility shifts on analysis of exon 5 (Fig. 3*B*, lanes 2 and 3). Tumors 3 and 21 showed mobility shifts on analyses of exons 6 and 7, respectively (Fig. 3*B*, lanes 1 and 4). The wild-type allele was detected in all these tumors.

DISCUSSION

In this study, we found a single-base-pair substitution of p53 in cDNA of SCC131. This mutation was in conserved region III. Furthermore, we observed the absence of mRNA from the wild-type allele. SSCP analysis of the genomic DNA of the p53 gene clearly demonstrated the absence of the wild-type allele. These gene alterations suggest that the p53 gene is involved in rat Zymbal gland tumorigenesis or acquirement of preferential cell growth *in vitro*.

To determine whether p53 gene mutations are relevant to *in vivo* carcinogenesis in the Zymbal gland, we analyzed the p53 gene mutations of primary Zymbal gland tumors induced by IQ. Four of 15 tumors were found to have mutations (Table 2). We analyzed the region from nt 307 to nt 881, which includes all the hot spots for mutations found in human carcinomas, but some tumors may have had mutations outside this region. The sensitivity of this method is very high; it allows detection of a minor constituent (about 3%) of mutated DNA (37), although all the positive cases detected in this study gave thick bands of shifts. However, we could not exclude false negative results by this method due to the absence of a band shift of a mutated DNA (38).

One of the four mutations detected was a single-base-pair deletion at codon 255 or 256, with the appearance of a termination codon within 276 base pairs downstream. Another mutation was present in conserved region III (codon 174). The other two mutations (codons 156 and 214) were outside conserved regions, but at scattered conserved residues. About 30% of all p53 mutations of human cancers are also reported to be located at scattered conserved residues (39), so it is very likely that the mutations at scattered conserved residues found in rat Zymbal gland carcinomas play some role in carcinogenesis in rats as well as humans.

Our data also suggest that the levels of mRNA from the wild-type p53 gene were much lower than those of the mutated alleles in three of these four tumors, but the wild-type allele was detected in all four of these tumors. Possibly the transcription of the wild-type p53 was impaired in all of these tumors except tumor 19, and the wild-type mRNA detected at low levels was produced by stromal cells coexisting with tumor cells. Another possibility is that the alleles that showed no band shift on genomic PCR-SSCP of exons 5–7 might have mutations in regions other than the coding sequence, as suggested in the case of Lu65, a large cell lung carcinoma cell line (38). A mutation of the p53 gene in the intron of the splicing site would reduce the level of p53 mRNA.

The frequency of *ras* mutations was found to be 100% (6/6) in IQ-induced rat Zymbal gland squamous cell carcinomas (M. Takahashi, personal communication). Thus it is conceiv-

Table 2. p53 gene mutations in a rat cell line of Zymbal gland tumor (SCC131) and primary tumors (4 of 15) induced by IQ

Sample	Codon	Nucleotide change	Amino acid change
SCC131	171	GTG → ATG	Val → Met
Tumor 3	214	GTG → TTG	Val → Leu
Tumor 5	174	TGC → TTC	Cys → Phe
Tumor 19	156	CGT → GGT	Arg → Gly
Tumor 21	256	GAA (deletion)	Frameshift

able that *ras* gene mutations are the primary event in carcinogenesis of rat Zymbal gland squamous cells and that a p53 gene mutation is a secondary or later event.

No histological differences were seen between carcinomas with or without p53 gene mutations, suggesting that p53 gene mutations do not affect their histology. Possibly other oncogenes or tumor suppressor genes with functions similar to the p53 gene had mutations in well-differentiated carcinomas in which the p53 gene was not mutated.

Two of the mutations found in IQ-induced Zymbal gland tumors were G → T transversions, one was a C → G transversion, and one was a deletion of a G·C base pair. Thus a guanine base was involved in all of these mutations. As the main IQ–DNA adduct formed *in vitro* is *N*-(deoxyguanosin-8-yl)-IQ (40), the mutations found in these Zymbal gland tumors may be induced by covalent binding of IQ to a guanine base in the p53 gene. This possibility is supported by the finding by Kudo *et al.* (41) that Ha-*ras* was activated in rat Zymbal gland tumors induced by IQ and that there were guanine → pyrimidine mutations in three of four positive cases. In contrast, the mutation found in the SCC131 cell line, which was established from a spontaneous tumor, was a G → A transition. IQ is also known to induce frameshift mutations at high frequency in *S. typhimurium*, and the G·C base pair deletion found in tumor 21 may have been due to covalent binding of IQ to a guanine base at or around the mutated site, which is present within the sequence CGCTGG.

Other chemical carcinogens have been proposed to induce specific mutations in oncogenes (42). We found that 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) specifically induced a G → T transversion at the second letter of codon 13 of Ha-*ras* in mouse forestomach squamous cell carcinomas (43). This specific mutation by MeIQ was also detected in rat Zymbal gland squamous cell carcinomas (41). A recent report showed frequent G·C → T·A transversions at the third letter of codon 249 of the p53 gene in human hepatocellular carcinomas in China and southern Africa, where both hepatitis B virus and aflatoxin B_1 are risk factors (44, 45). Furthermore, there is a report that the codon 249 mutation in the p53 gene was not found in hepatocellular carcinomas of patients not exposed to high levels of aflatoxin B_1, suggesting that the codon 249 mutation is specific to aflatoxin B_1 (46). The present study suggested that IQ induces mutations specifically at guanine bases, but not at any specific base. To our knowledge, this is the first report on the mode of p53 gene mutations in *in vivo* tumors that were induced by a single chemical compound.

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