Genetic instability and p53 mutations in metastatic foci of mouse urinary bladder carcinomas induced by N-butyl-N-(4-hydroxybutyl)nitrosamine

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In a variety of human malignancies, alteration of the p53 tumour suppressor gene is known as a significant indicator of late progression events including invasion and metastasis, with a possible close relationship to genetic instability. Mutational analysis of the p53 and H-ras genes was performed for 10 pairs of N-butyl-N-(4-hydroxybutyl)nitrosamine-induced invasive mouse urinary bladder carcinomas and metastatic foci. p53 Mutations were found in nine of 10 (90%) primary carcinomas and seven of 10 (70%) metastatic foci. A total of eight p53 mutations in primary carcinomas were common in metastatic foci in six pairs. Additional p53 or H-ras mutations which were not identified in the primary carcinomas were found in three metastatic foci. Evaluation of the allelic distribution of the p53 mutations using RT-PCR, PCR and subcloning, further indicated possible intra-tumour genomic heterogeneity or excess copy numbers of the p53 gene due to genetic instability. Overall, p53 alterations were frequent in mouse urinary bladder carcinomas demonstrating progression. The results suggest that genetic instability might underlie generation of additional genetic alterations in this animal model.

Introduction

Cancer invasion and metastasis directly affect the prognosis of patients, but only little information is available regarding the underlying mechanisms. Detailed analyses of multistage carcinogenesis at various organ and tissue sites have shown that alterations of the p53 tumour suppressor gene may play a significant role in neoplastic processes (1), particularly during progression. Initial evidence for this was provided by Sidransky et al. (2) who described p53 alterations to be related to human brain tumour clonal expansion and progression. During colon carcinogenesis, for which stepwise genetic alterations have been relatively well-characterized (1), p53 defects generally occur as a late event (3,4). Recent findings have also indicated a possible participation of p53 alterations in very late stages of development of other tumours (5,6), for which no relevance to p53 had earlier been concluded. Oda et al. (6) have found frequent p53 alterations limited to sarcomatous components of renal cell carcinomas. In human urinary bladder cases, p53 alteration is frequent in invasive and/or high-grade transitional cell carcinomas (TCCs*) (7–9) and Miyao et al. (10) have described both p53 mutation and loss of heterozygosity (LOH) of chromosome 17p, where the p53 gene is located, occurring before metastasis with common changes in primary carcinomas and metastatic foci.

Wild-type p53 serves as a critical regulator of a G1 cell cycle checkpoint following exposure of cells to DNA-damaging agents (11). If this check becomes defective, cells containing heritable DNA alterations may be allowed to enter S phase without adequate repair, so that genetic changes are ‘printed’ in their progeny. As a result, p53 insufficiency may accelerate the accumulation of other genetic alterations, such ‘genetic instability’ facilitating further progression of neoplastic disease (7,8,12).

In general, human beings are continuously exposed to a multitude of carcinogenic etiological or modifying factors in the environment, so that the accumulation of genetic alterations is complex. On the other hand, only limited factors are operating during experimental carcinogenesis so that investigations of induced tumours allow a finer focus on the genetic alterations which are responsible for changes in biological behavior.

Investigations concerning invasion and metastasis have in many cases been restricted by the lack of appropriate in vivo animal models, metastatic carcinomas being rare in these models as compared to the human case. However, the N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN)-induced urothelial carcinogenesis model in male NON/Shi mice, which demonstrate spontaneous hydronephrosis at an incidence of ~30%, is very useful for investigating the mechanisms of invasion and/or metastasis (13,14). Using this mouse model, we recently found p53 alterations to be frequent (14 out of 18 tumours; 78%) in invasive urinary bladder carcinomas without detectable metastatic foci, whereas ras oncogene alterations were infrequent (15). In the present study, a total of 10 primary urinary bladder carcinomas and paired metastatic foci were individually evaluated for p53 and H-ras abnormalities. To elucidate the transcriptional capability of the p53 gene, with and without mutations, the reverse transcription-polymerase chain reaction (RT-PCR) and subsequent single strand conformation polymorphism (SSCP) analysis were performed. The allelic distribution of p53 mutations was also examined using subcloning from amplified fragments of p53 cDNA.

Materials and methods

Production and isolation of tumours

BBN-induced urinary bladder carcinomas using male NON/Shi mice (Aburaih Lab. of Shionogi Co., Shiga, Japan) were produced and pathologically evaluated as previously described (15) with minor modifications. Briefly, mice were treated with BBN in drinking water at as high a concentration as they could tolerate, which ranged between 0.05–0.3%. After the completion of BBN treatment for 8–12 weeks, mice were maintained without any chemical
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supplement. Mice were killed in moribund condition under ether anesthesia between 14 and 23 weeks of the experiment. A total of 10 animals harbouring both urinary bladder carcinoma and metastatic foci were found at autopsy and used for further analysis. The largest primary carcinoma and representative metastatic foci were carefully washed fine scissors and immediately frozen separately, with special care to avoid mixture of carcinoma cells.

Nucleic acid preparation and RT-PCR

Nucleic acids were isolated from frozen tissues by the guanidine–thiocyanate–cesium chloride method (16) as previously described (15). After ultracentrifugation in cesium chloride gradients and subsequent extraction of the DNA layer, RNA pellets were gently washed twice with 70% ethanol, dissolved in distilled water, extracted with phenol–chloroform–isoamyl alcohol, and then ethanol was added, and the RNA stored at −80°C until used. Before cDNA synthesis, total RNAs were incubated with 10 U of RNase-free DNase I (Boehringer–Mannheim, Mannheim, Germany) and 20 U of RNase-inhibitor (Boehringer–Mannheim) at 37°C for 30 min. The RNA was ethanol precipitated following phenol–chloroform extraction and was resuspended in distilled water. First-strand cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (Superscript II, Gibco/BRL, Gaithersburg, MD).

First-strand cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (Superscript II, Gibco/BRL, Gaithersburg, MD). One microliter random hexamers (50 ng) were added to the RNA (5 µg in 8 µl distilled water), incubated at 70°C for 10 min and then quickly chilled on ice. Four microliters of 5× reaction buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 2 µl dithiothreitol, and 4 µl dNTP (2.5 mM) were added to the reaction mixture and incubated at 42°C for 2 min. After addition of 1 µl enzyme (200 U), the mixture was incubated at 42°C for 50 min, the reaction was inactivated by heating at 70°C for 15 min, dissolved in 80 µl dH₂O, and used as PCR. To confirm the presence of cDNAs, pairs for amplification for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were designed and used for PCR. Primer sequences used were: sense primer, 5′-ATC TTC TTG TTG AGT GCC AG-3′; and antisense primer, 5′-GTA GAG GTG GAT CAA GAA GG-3′.

Mutational analysis

PCR-SSCP analysis and direct sequencing for genomic p53 and H-ras genes were performed according to the previously described procedure (15). For amplification of p53 gene exon 8, the following oligonucleotide was employed as the antisense primer: 5′-GAG GTG ACT TTG GGG TGA AGC TCA ACA GGC TCC TC-3′. For several samples, to ascertain the results proved by PCR-SSCP analysis and direct sequencing, PCR products amplified from genomic DNA were subcloned using pCR-Script SK (+) cloning kit (Stratagene, La Jolla, CA), and five or more recombinant colonies were picked up and amplified in 3 ml of LB culture medium. Double-stranded DNA was extracted using Plasmid Mini Kit (QIAGEN, Chatsworth, CA) and was sequenced by a DNA Sequencing System (Model 373A, Applied Biosystems, Inc., CA) using designed primers, T3 or T7 promoter primers. For several cases, 5–20 clones were picked up, mixed together, cultured and sequenced.

To evaluate the transcriptional ability of p53 mutations, SSCP analysis was carried out for cDNAs. Sequences of primers used are: 4LU, 5′-ATC TTC TTG TTG AGT GCC AG-3′; 4RU, 5′-CCA AGT CTG TTA TGT GC-3′; 5RU, 5′-GAG GTG ACT TTG GGG TGA AGC TCA ACA GGC TCC TC-3′; and 6RD, 5′-GAT GAG GTG GAG GCA AGC TGA AGA TCT TC-3′. PCR amplification for SSCP analysis (RT-PCR-SSCP) was performed using primers 4LU and 6RD to yield a 244 bp product (exon 5), primers 5RU and 8RD to yield a 268 bp product (exons 6–7), and primers 7RU and 9LD to yield a 197 bp product (exon 8). Gels corresponding to the position of mobility-shifted bands were cut out, and transcribed mutations were confirmed by sequencing. For several cases, nested primer of 4LU (4RU) was used for sequencing.

Allelic distribution of p53 mutations

For carcinoma lesions which harboured two or more p53 mutations, PCR fragments were designed to include one or more exons of interest and amplified from cDNA using the primers listed above. PCR products were gel-purified into the vector (pCR-Script, Stratagene) and 10 or more efficient clones were extracted and sequenced. Presence or absence of mutations were confirmed for all exons included. Clones containing the same mutations as proved by RT-PCR-SSCP analysis were regarded as representative of mutated alleles.

Assessment for p53 mutations

As indicated above, we employed several procedures to identify p53 mutations. Initially, PCR-SSCP analysis was performed and was followed by direct sequencing for all candidate DNA extracted from representative mobility-shifts. When the alteration was confirmed by sequencing both strands, we regarded it as mutation. The cases for which peaks of sequencing results were equivocal, the exon of interest was amplified by PCR from genomic DNA and subcloned. If the same peaks suspected by SSCP analysis were detected in sequencing results from mixed culture or single clones, we regarded them as effective mutations. If two or more identical alterations were found in several clones, it was counted for mutation even though such alteration was absent in sequence results from SSCP analysis-derived mobility-shifts. For the assessment for mutations in p53 gene cDNAs using RT-PCR-SSCP analysis, the same indications were used.

Results

Histological findings

At autopsy, tumour masses were evident in the urinary bladder lumens along with metastatic foci in some animals. Incidences of urinary bladder and renal pelvic carcinomas were 180/240 (75%) and 17/240 (7%), respectively, and 34 of 180 urinary bladder carcinomas were metastatic. Metastatic foci were used for mutational analysis were located on the diaphragm in six animals and were observed as swollen lymph nodes within the peritoneal cavity in four animals (Table I). Of the 10 primary carcinomas in the urinary bladder six were squamous cell carcinomas (SCCs) and four were TCCs, three having mixtures of both cell types. Most primary carcinomas were bulky and harboured variant histological features, so that it was very difficult to determine which of multiple tumours were metastatic.

All the primary carcinomas were grade 3 (17), aggressively invasive, and diagnosed as stage 4 (18). As in our previous observations (15), four of nine carcinomas with SCC elements demonstrated proliferation of spindle cell components, and these were diagnosed as non-keratinizing, undifferentiated SCC. For all pairs, pathological features of metastatic foci were consistent with part of the primary carcinoma. Notably, SCC or spindle cell components selectively metastasized, even when the TCC component predominated in the primary carcinoma, histological diagnosis of the metastatic foci revealed nine to be SCCs with spindle cell components in four cases and a single TCC.

Mutational analysis for the p53 gene

According to the indications described in the Materials and methods, a total of 37 mutations were found in p53 gene exons 5 to 8 and H-ras exons 1 and 2 (Table II and Figure 1). A single mobility-shift in exon 8 (animal no. 8, metastatic focus) was the only mutation which could not be determined despite repeated sequencing analyses. All mutations within p53 gene exons 5 to 8 involved 1 bp substitution. Since the sense primer for the amplification for genomic p53 gene exon 5 included the initial 10 exon bases [codons 123 to 126, (15)], two alterations (metastatic focus of animal no. 7, codon 126, CCC→CCG; primary carcinoma of animal no. 9, codon 124, TCT→CCT) became evident only on RT-PCR-SSCP analysis. The 35 p53 mutations included two nonsense and five silent mutations (Table II).

Distribution of p53 mutations

As indicated in Table II, p53 mutations were found in nine of 10 (90%) primary carcinomas and seven of 10 (70%) metastatic foci. Eight p53 mutations were common in primary carcinomas and their metastatic foci. A total of 46 mutations of the p53 gene were detected in the present study (mutations in metastatic foci in these eight pairs were excluded) and in our previous work (15), and only five mutation duplications (involving codons 148, 159, 201, 234 and 243) and two in triplicate (codons 202 and 257) were observed. Therefore the probability of two non-randomly selected p53 mutations being identical is 0.01063, so that the presence of identical mutations in
primary and metastatic lesions is very strong evidence of a clonal relationship. In this regard, the 10 pairs of primary and metastatic foci were classified into four categories according to the distribution pattern of the metastatic foci. Only in a single pair (animal no. 1) were no mutations found in either the primary carcinoma or the metastatic focus. In six pairs (animal nos. 2–7), the mutations detected in metastatic foci were common with those in the primary carcinomas. In contrast, for two pairs (animal nos. 9 and 10), mutations were not found in metastatic foci despite the presence of two or more mutations in the primary carcinomas. The lack of any abnormal sequencing signals in these two metastatic foci was confirmed by direct sequencing from genomic DNA.

**Analysis of aberrant splicing**

For animal no. 5, a single bp substitution at the second base of intron 7 was common to the primary and metastatic foci. These alterations were evident on PCR-SSCP analysis because the antisense primer for amplification for gene exon 7 was designed to include the initial 33 bases of intron 7. These alterations were evident on PCR-SSCP analysis because the antisense primer for amplification for gene exon 7 was designed to include the initial 33 bases of intron 7.

**Table I.** Histological appearances of mouse urinary bladder carcinomas and their metastatic foci

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Primary urinary bladder carcinoma</th>
<th>Metastatic focus</th>
<th>Grade/Stage</th>
<th>Histology</th>
<th>Other foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TCC* &gt; SCC</td>
<td>Diaphragm</td>
<td>G3/T4</td>
<td>SCC</td>
<td>Lung, mesenteric L.N.</td>
</tr>
<tr>
<td>2</td>
<td>TCC</td>
<td>Lumbar L.N.</td>
<td>G3/T4</td>
<td>TCC</td>
<td>Lung</td>
</tr>
<tr>
<td>3</td>
<td>TCC &gt; SCC</td>
<td>Diaphragm</td>
<td>G3/T4</td>
<td>SCC</td>
<td>Lung, mesenteric L.N.</td>
</tr>
<tr>
<td>5</td>
<td>SCC/spindle</td>
<td>Splenic L.N.</td>
<td>G3/T4</td>
<td>SCC (spindle)</td>
<td>Lung, diaphragm, hepatic L.N.</td>
</tr>
<tr>
<td>6</td>
<td>SCC</td>
<td>Diaphragm</td>
<td>G3/T4</td>
<td>SCC</td>
<td>Lumbar L.N., hepatic L.N.</td>
</tr>
<tr>
<td>7</td>
<td>SCC</td>
<td>Diaphragm</td>
<td>G3/T4</td>
<td>SCC</td>
<td>Pancreas</td>
</tr>
<tr>
<td>8</td>
<td>SCC/spindle</td>
<td>Diaphragm</td>
<td>G3/T4</td>
<td>SCC</td>
<td>Liver, pancreas, mesenteric L.N.</td>
</tr>
<tr>
<td>9</td>
<td>SCC</td>
<td>Pancreas</td>
<td>G3/T4</td>
<td>SCC</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>SCC/spindle</td>
<td>Hepatic L.N.</td>
<td>G3/T4</td>
<td>SCC (spindle)</td>
<td></td>
</tr>
</tbody>
</table>

*Predominance is indicated for carcinomas with mixtures of the two histological phenotypes.

**Table II.** p53 and H-ras gene mutations in mouse urinary bladder carcinomas and their metastatic foci

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Primary urinary bladder carcinoma</th>
<th>Metastatic focus</th>
<th>Gene</th>
<th>Exon/ codon</th>
<th>Codon</th>
<th>Base change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>none</td>
<td>none</td>
<td>p53</td>
<td>7</td>
<td>252 ATC → ACC Ile → Thr*</td>
<td>252 ATC → ACC Ile → Thr</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>p53</td>
<td>p53</td>
<td>p53</td>
<td>5</td>
<td>159 ATC → ACC Ile → Thr</td>
<td>159 ATC → ACC Ile → Thr</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>p53</td>
<td>p53</td>
<td>p53</td>
<td>7</td>
<td>239 TGC → AGC Cys → Ser</td>
<td>239 TGC → AGC Cys → Ser</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>p53</td>
<td>p53</td>
<td>p53</td>
<td>5</td>
<td>126 CCC → CGC Pro → Arg</td>
<td>126 CCC → CGC Pro → Arg</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>p53</td>
<td>int7</td>
<td>p53</td>
<td>7</td>
<td>257 TCC → TCT Ser → Ser</td>
<td>T → A (aberrant splicing)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>p53</td>
<td>5</td>
<td>p53</td>
<td>7</td>
<td>257 TCC → TCT Ser → Ser</td>
<td>135 GCG → GTG Ala → Val</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>p53</td>
<td>p53</td>
<td>p53</td>
<td>5</td>
<td>159 ATC → ACC Ile → Thr</td>
<td>159 ATC → ACC Ile → Thr</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>p53</td>
<td>5</td>
<td>p53</td>
<td>7</td>
<td>159 ATC → ACC Ile → Thr</td>
<td>159 ATC → ACC Ile → Thr</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>p53</td>
<td>5</td>
<td>p53</td>
<td>7</td>
<td>159 ATC → ACC Ile → Thr</td>
<td>159 ATC → ACC Ile → Thr</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>p53</td>
<td>5</td>
<td>p53</td>
<td>7</td>
<td>159 ATC → ACC Ile → Thr</td>
<td>159 ATC → ACC Ile → Thr</td>
<td></td>
</tr>
</tbody>
</table>

*Mutations which are common in both primary and metastatic foci are in bold.

Mutations in metastatic bladder carcinomas
exons 6, 7 (primers used were 5RU and 8RD) and exon 8 (primers 7RU and 9LD) also showed identical mobility-shifts in both primary and metastatic foci. Therefore, a putative 420 bp fragment including exons 6–8 was amplified from cDNA of both primary and metastatic foci using 5RU and 9LD primers, and then subcloning was performed. In several clones, despite no alteration in exon sequences, a 15 bp sequence which was identical to the initial 15 bases of intron 7 (with 1 bp substitution at second base T→A) was determined between exons 7 and 8, suggestive of aberrant mRNA processing. Since this bp substitution was within the ‘splice donor site; exon-G/GT-intron’ (19), mRNA processing was carried out to the next G/GT sequence of intron 7 (subsequent 15 bases).

By subcloning and sequencing analysis from the primary carcinoma of this animal, point mutations at codon 257 and 263 were proven to be on different alleles (Figure 2).

**RT-PCR-SSCP analysis and allelic distribution**

After cDNA synthesis, PCR was performed using GAPDH primers to confirm the presence of effective cDNA templates. Amplification was lacking for only one metastatic focus of animal no. 8 (data not shown). RT-PCR-SSCP analysis and subcloning were therefore performed for cDNAs of 10 primary carcinomas and nine metastatic foci. Of 25 p53 mutations identified by PCR-SSCP analysis, 17 were also confirmed by RT-PCR-SSCP and subcloning. Except in one animal, the presence or absence of mobility-shifts on RT-PCR-SSCP analysis appeared consistent with those of PCR-SSCP analysis, indicating that most mutated alleles had been transcribed. Only for animal no. 2 was no significant mobility-shift observed with RT-PCR-SSCP analysis of both the primary and metastatic foci despite a definite shift evident with PCR-SSCP.

In the present study, eight lesions proved to harbour two or more p53 mutations. Their distribution on p53 alleles is clearly of interest. Using PCR from cDNA encompassing one to four exons of interest and subsequent subcloning of PCR products, we tried to determine how many kinds of mutated p53 alleles exist within a single lesion, to allow prediction of the different mutant products and possible participation in intra-tumour genomic heterogeneity or numerical abnormalities of the p53 gene. The allelic distribution of p53 mutations could be clarified for four animals. Representative clones extracted from four primary and two metastatic foci showed to be on different alleles (Figure 2).

**Discussion**

We previously found p53 mutations in 14 of 18 (78%) mouse invasive urinary bladder carcinomas without detectable metastatic foci (15), a frequency similar to that reported in the literature for human invasive urinary bladder carcinomas (7–9,20). Immunohistochemically detected p53 overexpression in metastatic foci from human urinary bladder carcinomas has been described by Moch et al. (20), with a strong erbB-2 association linked to malignancy. Therefore, it is proposed that the prominent progression of urinary bladder carcinomas in this animal model might be related to the frequent p53 alterations, although what role(s) p53 might play in metastasis remains to be determined.

In the present study, the animals received close to the maximal tolerated dose of carcinogen and multiple transformation events might therefore have occurred. It is speculated that the large primary carcinomas encountered, with multiple
variants of \( p53 \) mutations, might have arisen through the coalescence of independently developing tumours. However, since most primary carcinomas with multiple metastatic foci were bulky and harboured variant histological features, it was very difficult to determine if multiple tumours were involved.

We recently performed a clonal analysis of multifocally-developing urothelial tumours using a chimeric mouse carcinogenesis model using BBN (Yamamoto et al., unpublished) and found that, although multicentric development of small tumours was observed in independent animals, large lesions consisted of a single clonal phenotype, indicating predominant clonal expansion. Accordingly, it is possible that multiple variants of \( p53 \) mutations within a single lesion observed in the present study were due to intra-tumour genomic heterogeneity or excess copy numbers of the \( p53 \) gene. As shown in Figure 2, three mutated \( p53 \) alleles were extracted from each of four lesions. Possible induction of tumour genomic heterogeneity due to genetic instability was recently described by Sauter et al. (21), with aneuploidy being frequent in human urinary bladder carcinomas with both \( p53 \) deletions and overexpression. Moreover, they observed marked genomic heterogeneity in metastatic lesions.

In the present study, carcinogen treatment was limited to the initial 8–12 weeks of the experiment and most animals were then maintained without any carcinogen supplement for >5 weeks (average of 12 weeks). Accordingly, metastasis would most likely have occurred during this post-treatment period. The explanation of additional \( p53 \) mutations in metastatic foci which were not present in the primary lesions is thus difficult. Multiple transformation by heavy carcinogenic insult may have resulted in multiple \( p53 \) mutations within multiple cells. So that novel mutations in metastatic foci from different lesions could have been caused by the initial carcinogen treatment. In support of this hypothesis, the spectrum of these mutations, which may represent a ‘genetic footprint’ of the carcinogen, was found to be the same in metastatic foci as in primary carcinomas and in those of our previous study (15), with a high proportion of transitions and transversions at T:A pairs. On the other hand, since clonal expansion might occur at each stage of tumorigenesis as previously proposed by Nowell (22) and the histological consistency between primary and metastatic foci of the present study supports this idea, so that \( p53 \) mutations found in metastatic foci might have been present in small subpopulations of the primary carcinomas and therefore not detectable.

In human urinary bladder cases, most genetic alterations in metastatic foci are shared in common by the primary carcinomas (10). However, cumulative \( p53 \) mutations are found to occur during the progression stage (2,3,5,6). Thus another explanation for the additional \( p53 \) mutations in metastatic foci of this animal model is through genetic instability. Although RT-PCR and subcloning analysis was not available for the metastatic foci of animal no. 8, the eight additional mutations observed were highly suggestive of abrogation of the genome-stabilizing system. It is generally accepted that a cell with genetic instability is prone to additional genetic alterations, so that this is one of the most significant causative factors for development of invasive and metastatic behaviour (12). Promutagenic lesions in this animal model induced by BBN are unknown. Therefore, further examination concerning the mechanisms of \( p53 \) gene mutation are necessary.

Likely explanations for the fact that mutations could not be determined in two metastatic foci (animal nos 9 and 10) despite three genetic alterations in the primary carcinomas are: (i) the mutated allele was lost during the process of metastasis; (ii) one or more \( p53 \) alleles were already lost before metastasis with decreased functional wild-type \( p53 \) protein; (iii) subpopulations without any \( p53 \) mutation metastasized; (iv) a smaller tumour than the large tumour which we evaluated as the ‘primary tumour’ had metastasized; or (v) genetic alterations in the \( p53 \) gene exist which cannot be determined by our methods. For the first and second instances, several studies have shown that \( p53 \) abnormalities are correlated with gene amplification (11,12), allelic loss, and other chromosomal changes (12). Thus examination of the genetic status of metastatic foci in this model is warranted. We are presently investigating allelic imbalance of the \( p53 \) gene in invasive and metastatic mouse urinary bladder carcinomas using F1 hybrid mice. The fourth possibility is unlikely because the histological appearances of the primary tumours and metastatic foci were consistent. For the fifth, the probability of false-negative results can be lessened by using a variety of procedures.

In animal no. 5, a point mutation at the second base of intron 7 resulted in alteration of the splice donor site and consequent aberrant splicing during mRNA processing as became evident on RT-PCR-SSCP and subcloning. \( p53 \) Defects due to aberrant mRNA processing in an animal urinary bladder carcinogenesis model have previously been reported by Asamoto et al. (23). In contrast to the frequent \( p53 \) mutations in our mutational analyses of urinary bladder lesions (15), \( ras \) gene alterations occurred at low frequency. A novel \( ras \) mutation at codon 44 which we previously reported (15) was also found in the present study. However, its significance, if any, remains unknown.

A large body of mutation data for the latest stages of carcinogenesis, including transformation toward more malignant status (2,6) or metastatic potential (3,20,21) has accumulated, the results demonstrating alteration in the \( p53 \) tumour suppressor gene to be the most common and frequent event in a variety of types of neoplasia. Clonal expansion of subpopulations with \( p53 \) mutations might participate, as indicated by the present study. The tumour-suppressor activities of \( p53 \) have caused this protein to be called the ‘guardian of the genome’ (24). Mutational inactivation of this guardian role may allow the affected cells themselves to trigger clonal expansion and genetic instability. Not only \( p53 \) defects, but also other factors might be necessary for acquisition of additional genetic alterations. Further investigations are now required to identify other factors responsible for maintaining genetic instability. The present experimental animal model appears particularly suitable for this purpose.

Acknowledgements

We thank Dr Yoshihisa Yano (Second Department of Biochemistry, Osaka City University Medical School, Osaka, Japan) for his guidance and suggestions. This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan.

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Received on February 10, 1997; revised on June 3, 1997; accepted on June 26, 1997.