Clonal analysis of urothelial carcinomas in C3H/HeN\(\rightarrow\)BALB/c chimeric mice treated with \(N\)-butyl-\(N\)-(4-hydroxybutyl)nitrosamine

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The histological background for multifocal and metachronous development of urothelial carcinomas remains equivocal, although accumulated genetic evidence suggests monoclonal origin of multiple urothelial carcinomas. Clonal development of various preneoplastic and neoplastic urothelial lesions of C3H\(\rightarrow\)BALB/c chimeric mice induced by \(N\)-butyl-\(N\)-(4-hydroxybutyl)nitrosamine (BBN) was immunohistochemically investigated using a C3H strain-specific antibody. All tumor masses induced in the mice treated with 0.05% BBN for 20 weeks were composed of neoplastic cells of a single parental type, which is indicative of monoclonal lesions. Three of 10 animals harbored two or more separate carcinomas of different clonal type, which is indicative of multicentric development applicable in this model. Using DNAs derived from urothelial carcinomas and tumor-adjacent urothelium of chimeric mice, polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis and direct sequencing were performed. The clonality of several pathological lesions could be genetically confirmed using polymerase chain reaction to confirm the clonal origin of all cells in carcinomas as well as in pre- and non-neoplastic lesions. In the present study, these chimeric mice were treated with a specific urothelial carcinogen, \(N\)-butyl-\(N\)-(4-hydroxybutyl)nitrosamine (BBN), and the clonality of each urothelial carcinoma was examined. The clonal origin of several pathological lesions could be genetically confirmed using polymerase chain reaction-simple sequence length polymorphism (PCR-SSLP) analysis (12,13), and an evaluation of alterations of the \(p53\) tumor suppressor gene was included. Mouse urothelial

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carcinomas induced by BBN are characterized of non-papillary, high grade phenotype. The aims of this experiment were to (i) determine the clonal origin of multifocal urothelial carcinomas, whether monoclonal or multicentric, (ii) analyze the proposed model of histopathological genesis of non-papillary type carcinomas [from dysplasia to carcinoma in situ, (CIS) to invasive cancer] (9), and (iii) determine the role of p53 mutations in uterine carcinogenesis.

Materials and methods

Production of chimeric mice

C3H and ICR mice were purchased from Charles River Japan Inc. (Karagawa). BALB/c were from CLEA Japan, Inc. (Tokyo). All animals, including chimeric mice, were housed in plastic cages on hardwood chips in an air-conditioned room with a 12-h light-dark cycle. They were fed on basal diet (Oriental NMF, Oriental Yeast Co., Tokyo) and allowed access to tap water ad libitum.

C3H-BALB/c chimeric mice were produced by an aggregation procedure (14). First, eight-cell stage embryos of C3H and BALB/c strains were collected by oviduct flushing, and the zona pellucidae were removed by enzymatic digestion with pronase. When aggregated embryos of each genotype had reached the blastocyst stage, they were surgically transferred into the uteri of pseudopregnant ICR foster mothers. Chimeric animals showing the distinct chimeric coat color pattern were used in the analysis.

Experimental protocol

A total of 14 (11 male and 3 female), 7-week-old chimeric mice were divided into two groups; four male mice for group 1, and nine males and three females for group 2 (animal nos 1–10). Ten C3H and eight BALB/c mice were also examined (groups 3 and 4, respectively). At the beginning, animals were administered 0.05% BBN (Tokyo Kasei Kogyo Co., Ltd, Tokyo) in their drinking water for 6 and 20 weeks for groups 1 and 2–4, respectively. After the completion of the BBN treatment, they were maintained without any chemical supplement. Animals of group 1 were killed at week 20. Most animals in groups 2–4 were killed in a moribund condition between weeks 20 and 28, and the remainder were killed at week 30. Several chimeric mice without BBN treatment (range 8–20 weeks old) were evaluated as untreated controls.

Histological and immunohistochemical analysis

All animals were killed under ether anesthesia and the urinary tract and other organs were immediately removed and processed for fixation. Tissues of chimeric mice were initially fixed by microwave irradiation for 20 min at 40°C in phosphate-buffered saline (PBS) and then with ice-cold 95% ethanol plus 1% acetic acid for 6–10 h. They were then embedded in polyester wax and all animals were killed under ether anesthesia and the urinary tract and other organs were immediately removed and processed for fixation. Histological and immunohistochemical analysis was performed as previously described (15).

Histological classification of urothelial lesions

Histological classification of urothelial lesions was classified into four categories, namely, simple hyperplasia, dysplasia, CIS and (papillary or non-papillary) carcinoma (15,16). Within CIS lesions, minimal invasion was frequently observed and we defined the lesion as ‘CIS with microinvasion’. Carcinomas were classified as TCC or squamous cell carcinoma (SCC). Grading and staging of neoplastic lesions were histologically performed according to WHO (17) and TNM classifications (18), respectively.

Genetic analysis

Nucleic acids were extracted from polyester wax-embedded sections using a macrodigestion approach (19). Before extraction, each pathological lesion was diagnosed in H&E sections and the clonal origin was confirmed by CSA immunohistochemistry. Sections were dewaxed and air-dried, and using a fine needle, small pathological lesions were dissected out under the microscope with special care taken to avoid phenotypic heterogeneity. In particular, non-invasive lesions were dissected out so that they were distant enough from the invasive carcinoma. Tissues were collected in 20–50 µl of protein lysis buffer containing 0.1 mg/ml proteinase K. Samples were initially digested at 37°C overnight, then the enzyme was inactivated by heating to 90°C for 5 min and 1 µl of sample was used for PCR. PCR amplification was employed for eight dysplasia or CIS lesions (animals 1–4 and 6), 19 UBCs (animals 2–10) and six renal pelvic carcinoma (RPC; animals 4, 5, 7, 9 and 10).

To ascertain the clonal origin of each pathological lesion genetically, PCR-SSLP analysis using microsatellite polymorphisms was employed. Three sets of microsatellite primers (D1Mit17, D2Mit13, D11Mit14), which amplify sequences polymorphic between the C3H and BALB/c strains, were purchased from Research Genetics Inc. (Huntsville, AL). Using these primer sets and extracted template DNAs, PCR and gel electrophoresis were performed as previously described for PCR-single strand conformation polymorphism (SSCP) methods (20). Also, PCR-SSCP analysis and direct sequencing of p53 gene exons 5–7 were performed for several pathological lesions using the methods described previously.

Results

Histological appearance

No metastatic lesions were macroscopically or microscopically found in any animals, although UBCs and RPCs were observed in groups 2–4 (Tables I and II). In two animals of group 2 (nos 8 and 10), hydronephrosis caused by invasion by UBC was observed. Histological findings for chimeric mice of group 2 according to the criteria indicated in Materials and methods are indicated in Table II.

In all animals in group 1, simple hyperplasia was generally observed in the urinary bladder and renal pelvis, but no neoplastic lesions were found, presumably because of the insufficient length of carcinogen treatment. In contrast, animals in group 2 demonstrated both TCCs and SCCs (15) in their urinary bladder and renal pelvis, although benign papillomas were not present. No ureteral carcinoma was observed in any animal. In total, 20 UBCs (nine TCCs and 11 SCCs) and seven RPCs (four TCCs and three SCCs) were found in group 2. Most urothelial carcinomas observed in chimeric mice were high grade, non-papillary type (19 out of 27; 70%) and aggressively invasive. Fifteen of 27 carcinomas (56%) were diagnosed as greater than pT3. CIS or dysplastic lesions were usually observed adjacent to invasive carcinomas (Table II). Eight animals (nos 3–10) harbored two or more carcinomas in their urinary tract.

In non-chimeric animals in groups 3 and 4, all except one animal (group 3) harbored urothelial carcinomas (Table I). In animals of group 3 (C3H strain), UBC and RPC were found in 9/10 and 5/10 animals, respectively. As for group 4 (BALB/c strain), UBC and RPC were present in 8/8 and 1/8 animals, respectively. In total, the mean numbers of UBC and RPC per mouse were 1.1 and 0.7 in group 3, and 1.0 and 0.1 in group 4, respectively (Table I).

CSA-staining analysis

CSA-staining showed clear immunoreactivity in the cytoplasm with the difference between CSA-positive and negative cells being obvious. In organs other than the urinary tract, tissues were composed of epithelial cells of both parental types (C3H and BALB/c) distributed in a mosaic pattern. In chimeric animals without BBN treatment, relatively wide areas of urothelium were found to be of the same immunoreactivity (Figure 1A). Within these proposed clonal urothelial proliferating units (UPUs), basal and middle layers of the urothelium were always of the same clonal phenotype, however, some umbrella cells revealed distinct clonality. In animals of group 2, different immunoreactivity in flat epithelial lesions revealed their lateral clonal growth (Figure 1B).

Mean numbers of CSA-positive and -negative urothelial
### Table I. Induction of urothelial carcinomas in chimeric, C3H/HeN and BALB/c mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Strain</th>
<th>No. of animals examined</th>
<th>BBN treatment (weeks)</th>
<th>UBC Incidence (%)</th>
<th>No./animal&lt;br&gt;</th>
<th>RPC Incidence (%)</th>
<th>No./animal&lt;br&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C3H ↔ BALB/c chimera</td>
<td>4</td>
<td>6</td>
<td>0/0 (0)</td>
<td>0</td>
<td>0/0 (0)</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>C3H ↔ BALB/c chimera</td>
<td>10</td>
<td>20</td>
<td>10/10 (100)</td>
<td>1.9 ± 1.3</td>
<td>5/10 (50)</td>
<td>0.7 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>C3H tumor</td>
<td></td>
<td></td>
<td>8/10 (80)</td>
<td>1.6 ± 1.0</td>
<td>3/10 (30)</td>
<td>0.5 ± 1.0</td>
</tr>
<tr>
<td>3</td>
<td>C3H</td>
<td>10</td>
<td>20</td>
<td>9/10 (90)</td>
<td>1.1 ± 0.6</td>
<td>5/10 (50)</td>
<td>0.7 ± 0.8</td>
</tr>
<tr>
<td>4</td>
<td>BALB/c</td>
<td>8</td>
<td>20</td>
<td>8/8 (100)</td>
<td>1.0 ± 0.0</td>
<td>1/8 (12.5)</td>
<td>0.12 ± 0.35</td>
</tr>
</tbody>
</table>

*Mean ± SD.

### Table II. Histological appearances and p53 mutations in urothelial carcinomas of chimeric mice treated with BBN (Group 2)

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Sex</th>
<th>Lesion location</th>
<th>Histology</th>
<th>Clonal type</th>
<th>Grade/ stage</th>
<th>Morphological type&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p53 Mutation (mutated codon)</th>
<th>Adjacent urothelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>Bladder</td>
<td>TCC</td>
<td>C3H</td>
<td>G3/pTis</td>
<td>N</td>
<td>not determined (exon 5)</td>
<td>dysplasia (C3H)</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>Bladder</td>
<td>TCC</td>
<td>C3H</td>
<td>G3/pT1</td>
<td>N</td>
<td>none</td>
<td>dysplasia (C3H)</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>Bladder</td>
<td>TCC</td>
<td>C3H</td>
<td>G3/pT1</td>
<td>N</td>
<td>148</td>
<td>dysplasia (C3H)</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>Bladder SCC&gt;TCC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>BALB/c</td>
<td>G3/pT1</td>
<td>N</td>
<td>133,251</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CIS and dysplasia (C3H)</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>Pelvis SCC</td>
<td>C3H</td>
<td>G3/pT3</td>
<td>P&lt;sup&gt;e&lt;/sup&gt;</td>
<td>159</td>
<td>CIS (BALB)</td>
<td>176</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>Bladder</td>
<td>TCC</td>
<td>C3H</td>
<td>G3/pT3</td>
<td>N</td>
<td>189</td>
<td>dysplasia (C3H)</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>Bladder SCC</td>
<td>C3H</td>
<td>G3/pT4</td>
<td>P + N</td>
<td>238,251</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>dysplasia (C3H)</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>Pelvis SCC</td>
<td>BALB/c</td>
<td>G2/pT2</td>
<td>N</td>
<td>none</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>none</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>Bladder SCC</td>
<td>C3H</td>
<td>G3/pT3</td>
<td>P&lt;sup&gt;e&lt;/sup&gt;</td>
<td>131</td>
<td>none</td>
<td>dysplasia (C3H)</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>Pelvis SCC</td>
<td>C3H</td>
<td>G3/pT4</td>
<td>P + N</td>
<td>148&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CIS (C3H)</td>
<td>NE&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>M, male; F, female.

<sup>b</sup>P, papillary type; N, non-papillary type.

<sup>c</sup>Microdissection of non-invasive lesions were performed from the areas where it is significantly far from the invasive lesions.

<sup>d</sup>Predominance is indicated for tumors with mixtures of the two histological phenotype.

<sup>e</sup>ND, not determined because of the expansion of tumor growth.

<sup>f</sup>Alteration at the second base of intron 7 (GTA to GGA).

<sup>g</sup>NE, not evaluated.

<sup>h</sup>These two mutations are identical (CCT to TCT).

carcinomas (TCC + SCC) per animal in group 2 were 2.1 and 0.6, respectively. All carcinomas were composed of neoplastic cells of a single parental type (Figure 1C), which is indicative of a monoclonal lesion. Three of 10 animals in group 2 (nos 7, 8 and 10) harbored two or more independent carcinomas of different clonal phenotype (Table II). Multicentric carcinoma development in the urinary bladder was represented in animal no. 8 (Figure 1C). Three independent carcinomas were located within the same vicinity and each carcinoma accompanied widely-distributed CIS lesions of the same clonality.

CSA-staining of BBN-treated chimeric urothelium without carcinoma lesion involvement demonstrated homogeneous positive or negative immunoreactivity, which suggests lateral clonal expansion of the clones with selective growth advantage. Generally, CSA-positive urothelium, which indicates an origin from the C3H parental type, predominated. As indicated in Table II, all but a single invasive carcinoma were surrounded...
by the pre- and neoplastic lesions (CIS or dysplasia) of the same clonal phenotype. Notably, in several cases, CIS lesions adjacent to invasive carcinomas demonstrated frequent mitotic figures and foci of microinvasion (Figure 1C), which is strongly suggestive of lateral clonal expansion of the cells with invasive potential. In most cases, separate tumors of the same clonal phenotype coexisting within the large areas were of the same parental phenotype.

**PCR-SSLP analysis and p53 mutations**

Using three microsatellite markers, which are polymorphic between the two parental strains, clonalities of individual carcinomas were genetically confirmed by PCR-SSLP analysis (12,13). Lesion-derived DNA amplified by PCR presented strain-specific mobility shifts that were similar to the parental strains (data not shown). All data were consistent with the results of CSA-staining.

According to our previous results concerning mouse urinary bladder carcinomas induced by BBN (20,21), p53 mutations were concentrated in exons 5–7 and no evident hotspot was found; the probability that two randomly selected p53 mutations being identical is nearly 1%. Using DNAs derived from urothelium adjacent to seven tumors (dysplasia or CIS) and 26 carcinomas of mice in group 2, PCR-SSCP and direct sequencing were performed for p53 gene exons 5–7. As indicated in Table II, p53 mutations were identified in four of 11 (36%) non-invasive lesions (dysplasia, CIS and pTa tumor) and 13 of 22 (59%) invasive carcinomas. Although p53 mutations were frequently found in UBCs (13 of 20, 65%, including a single CIS of animal no. 1), RPCs harbored less frequent alterations (two of six, 33%).

For five cases, SSCP analysis and direct sequencing were performed for both invasive carcinomas and non-invasive lesions, and no identical mutation was found. However, two non-invasive lesions contained distinct p53 mutations (animal nos 4 and 6). Only in a single case (animal no. 10) were identical p53 mutations (codon 148, CCT→TCT) found in separate UBCs (Figure 2); one was a papillary SCC at the dome of the urinary bladder and the other was an invasive SCC located at the base. However, the former was found to harbor an additional mutation in exon 7 (codon 237, AGC→AGA), which has not been detected in other carcinomas.
Discussion

In the present study, two conclusions concerning the development of mouse urothelial carcinomas became evident. Firstly, an individual urothelial tumor mass is made up of cells of the same genetic background (monoclonal), which is supported by the CSA-staining and PCR-SSLP analysis. Secondly, multiple urothelial tumors that developed in the present mouse model are of different clonal origin. Three of 10 chimeric mice harbored two or more urothelial carcinomas of different parental genotypes, which indicates that these carcinomas originated from independent precursor cells.

The concept of a ‘field change’ has long been used to describe the histological appearances of urothelial tumors (1,4) because large areas of the urothelium are usually involved with dysplasia and CIS concomitant with TCC. Multifocal development of urothelial tumors makes the implications of this concept more complex. Two distinct explanations for the field change have been proposed: one is independent transformation of many epithelial cells because of strong carcinogen insult; and the other possibility is migration or expansion of foci of transformed urothelial cells (6). We attempted to describe clonal development of the field changes within the urinary tract, using the chimeric mouse carcinogenesis model. Here we clearly demonstrate that carcinogen exposure can induce multiple urothelial transformations. Genetic evidence proved that the DNAs derived from different pathological lesions harbored distinct alterations of the \( p53 \) tumor suppressor gene, although it should be stressed that, in general, the carcinogen exposure in experimental animal models is much greater than in humans. Spruck et al. (9) have suggested the existence of independent alterations of the \( p53 \) gene in urothelial cell populations in the same patient. Moreover, we recently found different \( p53 \) mutations from different urothelial lesions occurring in individual patients who were living in radiocontaminated areas of Ukraine (unpublished data). These findings imply that multiple transformation events by strong carcinogen exposure is applicable in humans and that field changes caused by independent transformations might occur.

In contrast with our results, some evidence for human urothelial carcinomas has accumulated (6–8) showing that separate tumors sampled from the same patient harbor the same genetic alterations, which suggests that multifocal urothelial tumors can arise from a single initial clone. In our experiment using chimeric mice, most urothelial tumors were of the non-papillary type (19 out of 27; 70%) and 15 of them (56%) were diagnosed as having a higher stage than pT3. In contrast, in the reports of Sidransky et al. (6) and Habuchi et al. (7), the incidences of tumors lower than pT2 were 92% (12 of 13) and 64% (7 of 11), respectively, presumably most being of the papillary type. Spruck et al. (9) have suggested the participation of two molecular pathways in urinary bladder carcinogenesis. Therein, \( p53 \) alterations appear to occur early in CIS and dysplasia prior to non-papillary invasive lesions but late in papillary TCCs. It can therefore be suggested that different types of clonal expansion may arise in the two histopathological phenotypes. In our results, identical \( p53 \) mutations were determined in a single UBC pair, which had papillary features, although they were SCCs. Papillary SCC is an uncommon phenotype in human UBCs except in areas of endemic schistosomiasis (22). Thus, the mechanism of how papillary tumors originate from a single clone developing independently remains unclear.

Nowell (23) previously postulated that clonal development of cells that represent each step of the carcinogenesis process may consist of heterogeneous cell populations. Subsequently, clones that have acquired selective growth advantage or additional genetic alterations are expanded until they comprise the bulk of the tumor. In human urinary bladder cases, dysplasia or CIS lesions concomitant with bladder carcinomas commonly occur (1,5,24); however, genetic evidence demonstrating that these lesions are the remnant of the previous carcinogenesis stage is lacking. In the present study using C3H→BALB/c chimeric mice, histopathological evaluation of BBN-treated urothelium provided evidence for monoclonality of individual carcinomas as well as their adjacent preneoplastic lesions. Such topographical distribution of pathological lesions within a single putative UPU provides clear evidence for the progressive developmental model of non-papillary type urothelial carcinogenesis, starting with dysplasia or CIS with subsequent invasive carcinoma. During each step in the urothelial carcinogenic process, lateral clonal expansion might occur, in line with the model previously presented by Nowell (23). In particular, frequent mitotic figures and foci of microinvasion within CIS lesions are concentrated in the areas adjacent to the invasive carcinoma, which suggests lateral clonal expansion of cells of invasive potential. Harris and Neal (4) suggested that inflammatory changes in the urinary tract may facilitate the expansion of such clones. In addition, if there is any exposure to chemical carcinogen in the urine, resistant transformants may gain a growth advantage over sensitive populations. Certain preneoplastic populations themselves may intrinsically possess increased proliferative potential. It is possible that large, clonal, preconditioned urothelium might provide ‘background lesions’ for multifocal or metachronous urothelial carcinomas, as supported by clinical evidence (1,5). In particular, transurethral resection of tumors cannot resect completely the ‘background lesions’.

The relative homogeneity observed for the urothelia in chimeric mice without chemical supplement was in clear contrast to the mosaic patterns in other organs (11–13), which suggests that the basic UPU of chimeric mouse urothelium is relatively large. Tsai et al. (25) have recently demonstrated the monoclonality of macroscopic human urothelial patches in female patients. In the present study, biased occupation of C3H clones in urothelium of BBN-treated animals was observed; however, the underlying mechanism remains unknown. Sensitivity or resistance to carcinogen might differ between C3H and BALB/c clones, although carcinoma induction of their inbred parental strains (groups 3 and 4) did not differ. Also, biased carcinoma induction of C3H clones might correspond to their background areas. Whether separated tumors of the same clonal phenotype are derived from a single precursor cell remains unclear. Although \( p53 \) mutations were different in all except one case, we cannot completely rule out the possibility of their monoclonality, because \( p53 \) alterations might not always be an initial event. In our results, distinct \( p53 \) mutations between non-invasive and invasive carcinoma were found in two cases, which indicates independent and/or divergent genetic alterations. Investigation and utilization of earlier genetic markers may facilitate our understanding in greater detail of clonal development. In the present study, the incidences of \( p53 \) mutations in invasive lesions (13 of 22; 59%) was similar to those in human high-grade, advanced UBCs (9,26), with mutations pre-existing in adjoining non-invasive lesions. It is postulated that the role of \( p53 \) mutations
in mouse urinary bladder carcinogenesis is to acquire a more malignant phenotype (20,21), especially with regard to invasive potential.

Overall, taking into account the evidence presented in this paper and that in the literature, the following implications can be derived for clonal development of urothelial carcinomas: (i) ‘field changes’ caused by a strong carcinogenic insult occur and lead to multiple transformations and independent development of tumors; (ii) individual tumors are clonal in their composition; and (iii) the potential tumor cells present around an exophytic tumor mass are the source of recurrence around the same genetic background. These findings support the conclusion that multiple urothelial tumors can be of either monoclonal or multiple clonal origin.

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