p53 Status in Multiple Human Urothelial Cancers: Assessment for Clonality by the Yeast p53 Functional Assay in Combination with p53 Immunohistochemistry

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Multifocal synchronous or metachronous tumor development is a common observation in human urothelial cancer cases. However, the underlying mechanism has remained obscure. We have employed a new tool to investigate the p53 gene status, the yeast p53 functional assay, in combination with immunohistochemistry in a total of 50 tumor samples from 32 cases with urothelial cancers, including 8 with multiple synchronous tumor development and 2 demonstrating metachronous tumors. p53 mutations were found in 13 cases (9 with missense mutations, 3 with deletion, 1 with splicing mutation) by the yeast p53 functional assay. p53 protein overexpression was seen in all 9 cases with missense mutations, but in only one of the 4 cases with nonsense mutations. Two tumors without p53 mutation also showed positive p53 immunoreactivity. Overall, p53 abnormalities including mutations and/or protein overexpression were found in 15 (47%) cases. p53 abnormalities were significantly more frequent in non-papillary and in high grade tumors. Loss of the wild type allele in addition to a p53 mutation was suggested in 8 of the 15 (53%) cases. All 4 cases with mutations in multiple synchronous tumors had identical p53 mutations in the separate urothelial cancers, strongly suggestive of monoclonality. The one case with multiple metachronous tumors, in contrast, was characterized by variation in the p53 status, indicative of different clonal origins. In conclusion, combined assessment for p53 status as used here (yeast p53 functional assay plus immunohistochemistry) may provide insights into the molecular mechanisms of urothelial carcinogenesis.

Key words: Urothelial cancer — p53 — The yeast functional assay — Clonal growth — Loss of heterozygosity

During the multi-step process of carcinogenesis, alteration of the p53 tumor suppressor gene is one of the most common observations in a variety of human malignant tumors.1, 2) In human urinary bladder cancers, it has been reported that p53 mutations are common in invasive and/or high grade tumors and a role in tumor progression has therefore been speculated.3) Human urothelial transitional cell carcinomas (TCCs) are classified into two distinct morphological subtypes; papillary and non-papillary.8) Papillary urinary bladder cancers, accounting for about 70% of all bladder neoplasms, are generally associated with better clinical outcomes than the non-papillary type, which are usually high grade and characterized by rapid progression and poor prognosis. Spruck et al.7) initially suggested the existence of two molecular pathways in the pathogenesis of urinary bladder TCCs, one featuring early occurrence of p53 abnormalities in carcinoma in situ (CIS) and/or dysplasia leading to non-papillary invasive lesions, and the other leading to rare and late occurrence in papillary TCC. In the former pathway, p53 mutation is associated with the loss of 17p allele in which the p53 gene is located, resulting in a complete loss of p53 function.3) We have recently demonstrated that cyclin D1 overexpression may be an alternative to p53 alteration in the pathogenesis of papillary TCCs.9)

Multifocal synchronous and metachronous development is commonly observed for human urothelial cancers,8) although the underlying mechanisms have remained unclear.10) Quantitative and qualitative molecular profiles of each tumor serve as the most useful means for studying the clonal origin, with an identical somatic mutation in different tumors strongly indicating monoclonality. Recently, a new tool for investigating p53 mutation has been established,11, 12) namely the yeast p53 functional assay. The assay tests the ability of human p53 to activate transcription in yeast. The transcription reporter is the ADE2 gene expressed from a p53-responsive promoter and colonies containing wild type p53 are white, while these containing mutant p53 are red. Since human p53 cDNA polymerase chain reaction (PCR) products can be cloned directly into the reporter yeast strain by homologous

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recombination without intermediate bacterial cloning steps, the percentage of red yeast colonies accurately reflects the mutant p53 mRNA content of the starting material. The assay can detect a mutant p53 in a minor fraction of tumor cell clones, facilitating clonal analysis. In addition, when a mutant content exceeds 50%, the tumor must be homozygous for the mutant allele, allowing analysis of loss of heterozygosity (LOH). To investigate p53 abnormalities in human cancers, the immunohistochemical approach has been frequently employed, because nuclear accumulation of p53 protein strongly suggests a mutational event that abrogates p53 degradation. Sarkis et al. suggested that immunohistochemical detection of p53 nuclear overexpression should be performed together with other molecular approaches. We therefore investigated p53 abnormalities in a series of human urothelial cancers using the yeast p53 functional assay and p53 immunohistochemistry in combination. The aims of this study were (i) to evaluate the genetic status of the p53 gene, and (ii) to determine the clonal origin of multifocal urothelial cancers.

MATERIALS AND METHODS

Tumor materials In the period from June 1995 and April 1997, surgical specimens were obtained from 32 patients (25 males and 7 females; aged from 40 to 83 and averaging 66 years old), who underwent surgery at the Department of Urology, Osaka City University Hospital, Osaka, Japan, and affiliated hospitals. Only a single frozen tumor specimen was available in each of 22 cases even though multiple tumors were sometimes present (2 cases, BT-11 and -14 had multiple tumors). On the other hand, frozen materials from multifocal lesions were available in 8 cases who received nephroureterectomy and/or cystectomy (M-1 to 8; multiple synchronous tumors).

The other two patients (M-9 and -10) received repeated resection or biopsies of urinary bladder cancers (multiple metachronous tumors). Eight samples were obtained by cold cup biopsy before transurethral resections. Ten of the 32 patients received systemic or intravesical chemotherapy before surgery (Table I). Immediately after the resection, tumor material was snap-frozen in liquid nitrogen and stored at −70°C prior to RNA extraction.

Remaining tumor materials as well as resected urothelial tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned and routinely stained with hematoxylin and eosin (H&E) for histological examination. Grading and staging of urothelial cancers were performed according to the criteria defined by the World Health Organization’s (WHO) International Classification of Tumors and with the staging systems recommended by the International Union Against Cancer, respectively. Yeast p53 functional assay RNA was extracted from frozen samples (trimmed to approximately 5×5 mm) using ISOGEN (containing phenol and guanidine thiocyanate, Nippon Gene, Toyama) according to the manufacturer’s instructions. The yeast p53 functional assay was performed as previously described with minor modifications. Briefly, RNA samples were reverse transcribed at 37°C using RNase H+ Moloney murine leukemia virus reverse transcriptase (Superscript II, Gibco/BRL, Gaithersburg, MD) and a 25 pM p53-specific primers (RT-113). Complementary DNA was amplified by PCR with recombinant Pfu polymerase (Stratagene, La Jolla, CA) and the P3 and P4 primers. Crude PCR products and a linearized p53-expression vector were co-transfected into the yeast reporter strain yIG397 and plated and grown at 30°C as previously described. To assess the temperature-sensitivity, all samples diagnosed as negative at 30°C assay were again assessed at 35°C. For samples with which significant percentages of red colonies were obtained, at least 4 independent yeast colonies were sequenced, as previously described. The existence of more than 4 clonal mutations within sequenced yeast colonies was the criterion for the final definition of mutation.

p53 immunohistochemistry A total of 55 paraffin-embedded tumor specimens from 32 patients were evaluated for p53 immunohistochemistry. In brief, after deparaffinization and blocking of endogenous peroxidase, antigen retrieval was routinely performed as previously described. The anti-p53 rabbit polyclonal antibody CM1 (1:500; Medac GmbH, Hamburg, Germany) was incubated overnight with specimens at 4°C. Staining was achieved using the Vectastain ABC-PO kit (Vector Laboratories, Burlingame, CA) and developed with the 3,3′-diaminobenzidine tetrahydrochloride (DAB) chromogen (Wako, Tokyo). Only nuclear localization of immunoreactivity was evaluated as positive. Assessment of the extent of nuclear reactivity was performed as described by Goto et al.: −, negative tumors; +, low expressers (1 to 9% immunoreactive tumor cells); +, moderate expressers (10 to 49% immunoreactive); and ++, high expressers (50 to 100% of tumor cells). For statistical analysis, tumors with more than 10% immunoreactive nuclei (+ and ++) were assessed as positive.

Statistical analysis All 2×2 tables were analyzed by the χ² test or Fisher’s exact probability test. P values less than 0.05 were considered statistically significant.

RESULTS

Yeast p53 functional assay It has been reported that the yeast p53 functional assay performed on clinical samples containing only wild type p53 gives 5 to 10% red colonies. In the present study, cases showing more than 10% red colonies were further subjected to sequence analysis. As shown in Tables I and II, a total of 50 assays
were performed on 32 cases and 19 tumors (14 cases, 14%) gave more than 10% red colonies. Only in a single urinary bladder cancer of case M-1, which showed 14% red colonies, was no clonal mutation found in the plasmids recovered from 4 independent yeast colonies, this therefore being assessed as negative for p53 mutation. Ten of the 18 tumors gave more than 80% red colonies, and were thus considered to have lost the wild type allele (LOH). Three tumors from two cases (BT-15 and M-6) had temperature-sensitive mutations (white colonies at 30°C but red at 35°C, Fig. 1).

Of the total of 14 mutations found in the 13 cases, 10 (71%) were single base-pair (b.p.) substitutions, 2 (14%) were frame-shift deletions, one (7%) was a frame shift insertion, and one (7%) was a skipping of exons 4 to 8. One (BT-5) of the b.p. substitutions at the splice donor of exon 6 (224 GAG → GAA) was considered to have resulted in a shift of the splice junction into intron 6 for 5 b.p.s, giving an apparent 5-b.p. insertion (224/225 GAG/GTT → GAAGTCTG). Of the 11 substitutions including case BT-5 (224 GAG → GAA), 8 (73%) were transitions, 6 being G:C to A:T, and 4 at CpG dinucleotides. G:C to T:A and G:C to C:G transversions accounted for 2 (14%) and one (7%), respectively. These proportions were similar to those reported by Xu et al.17) Two mutations were found in three tumors of case M-7 and sequencing of plasmids from 10 independent red colonies from these three tumors showed that 3 contained a mutation at codon 125, one contained a mutation at codon 211 and 6 contained both mutations, suggesting that intragenic recombination might have occurred between different p53 alleles in yeast.12)

Of the cases for which only a single tumor material was available (Table I), more than half (8 out of 15; 53%) had mutations, but no mutation was detected in 7 renal pelvic/ureteral cancers (P<0.02, χ2 test). Four out of 8 cases (50%) with multiple synchronous tumors had identical p53 mutations (M-5 to -8, Table II), indicating that the different tumors in each case had originated from a single clone.

Table I. Mutational Analysis of the p53 Gene in Urothelial Tumors by Yeast Functional Assay and Results of Immunohistochemistry

| Case no. | Age/sex | Tumor type | Yeast assay red colonies (%) | p53 mutations | p53 immuno-histochemistry |
|----------|---------|------------|-------------------------------|---------------|--------------------------|
| BT-1     | 65/M p, TCC, G1, pTa | 5.5 | None | \(224 \text{ GAG} \rightarrow \text{GAA}\) \(\rightarrow \text{GAAGTCTG} \rightarrow \text{GTT}\) Stop at codon 248 | – |
| BT-2     | 62/M p, TCC, G1, pTa | 6.5 | None | – | – |
| BT-3\(^c\) | 62/M p, TCC, G2, pT1 | 4 | None | – | – |
| BT-4\(^c\) | 68/M p, TCC, G2, pTa | 3 | None | – | – |
| BT-5\(^c\) | 66/M p, TCC, G2, pT1 | 22 | 6/7 | 224/225 GAG/GTT → GAAGTCTGGTT Stop at codon 248 | – |
| BT-6     | 82/M p, TCC, G3, pT1 | 41 | 7 | 226-227 GGCT → CGG CT | – |
| BT-7     | 53/M n, TCC, G3, pT4 | 4 | None | ++, hetero. | – |
| BT-8\(^c\) | 59/M n, TCC, G3, pT3 | 4.5 | None | – | – |
| BT-9     | 69/F n, TCC, G2, pT1 | 4 | None | – | – |
| BT-10\(^d\) | 61/M n, TCC, G2, pT3 | 24 | 6 | 220 TAT → TGT Thr → Cys ++, hetero. | – |
| BT-11\(^d\) | 74/M n, TCC, G3, pT1 | 97 | 5 | 132 AAG → AAC Lys → Asn + | – |
| BT-12\(^d\) | 69/F n, TCC, G3, pT1 | 82 | 5 | 176 TGC → TTC Cys → Phe +, scatter | – |
| BT-13\(^d\) | 68/F n, TCC, G3, pT3 | 96 | 7 | 248 CGG → TGG Arg → Trp + | – |
| BT-14\(^d\), BT-15\(^c\) | 83/M n, TCC, G3, pT4 | 99 | 5 | 175 CGC → CAC Arg → His +, hetero. | – |
| BT-15\(^c\) | 40/M n, ade, well, pT2 | 96 | 8 | 285 GAG → AAG Glu → Lys ++ | – |

Renal pelvic (RPT) and ureteral (UT) tumor

RPT-1 80/M p(r), TCC, G1, pTa 5.5 None
RPT-2 68/F p(l), TCC, G2, pT1 8.5 None
RPT-3 60/M p(r), TCC, G2, pT2 3 None
RPT-4 55/M p(r), TCC, G2, pT1 3 None
RPT-4 71/M p(l), TCC, G2, pT1 1.5 None
UT-1 58/M n(r), TCC, G3, pT2 8.5 None
UT-2 62/M p(r), TCC, G1, pT1 2 None

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a) p, papillary tumor; n, non-papillary tumor.
b) Hetero., tumor with heterogeneous immunoreaction; scatter, p53-positive cells were scattered.
c) Patients who received chemotherapy before surgery.
d) One of multiple synchronous tumors.
e) Temperature-sensitive p53 mutant.

p53 Mutation in Urothelial Cancers
In three cases (M-5 to -7), the percentages of red colonies were similar in independent tumors, suggesting the same allelic status of the $p53$ gene. On the other hand, although a mutation at codon 246 was identical in two tumor samples (BT-a and -b, case M-8), the percentages of red colonies were different; the urinary bladder cancer located at
the vesical neck (BT-a) gave 48% and the other cancer in the left lateral wall (BT-b) gave 81% red colonies, suggesting that the latter had lost the wild type allele (LOH). In one case of multiple metachronous tumors (M-9), that at the second operation (BT-b) gave 14% red colonies, while the first and third ones gave 6.5% and 2.5% red colonies, respectively. Sequencing of recovered plasmids showed a relatively large deletion (532 b.p.s encompassing exons 4 to 8, codons 106 to 282) in 6 of 8 red colonies. The second and third tumors (BT-b and -c) were immunohistochemically positive with a heterogeneous pattern and scattered positive nuclei (Fig. 2B). Therefore, the results suggested that the tumors in this M-9 case had different clonal origins.

**p53 immunohistochemistry** In a total of 32 cases evaluated for immunohistochemistry, 12 (38%) were assessed as positive for p53 accumulation. Interestingly, all tumors with single b.p. substitutions had a strong immunoreactivity (Fig. 2A; Tables I and II), while 3 of 4 cases with nonsense mutations (BT-5, -6 and M-5) were negative. In 6 tumors (BT-12, M-4 BT-a, -b and -c, M-9 BT-b and -c), 4 of which were devoid of *p53* mutation, positive nuclei

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**Fig. 1.** Yeast functional assay and subsequent sequence analysis. (a) Yeast functional assay for urinary bladder adenocarcinoma of case BT-15 gave 96% red colonies at 35°C, although assay at 30°C gave only 4% red colonies (temperature-sensitive mutation). (b) Sequence chromatograms of the same case showed a mutation at codon 285, GAG to AAG. The identical mutation was found in all of 4 red colonies examined.

**Fig. 2.** Different patterns of positive nuclear staining for p53. (a) Intensively stained nuclei of an invasive lesion assessed as a high expresser (++) (BT-10, ×250). (b) In this papillary tumor (BT-B of case M-9), positive nuclei were randomly distributed so that assessment was ‘scattered’ (×250).
were sparsely distributed throughout the tumor specimens (scattered pattern, Fig. 2B). The other four tumors (BT-7, BT-10, BT-14, M-6 RPT(rt)) showed heterogeneous staining patterns with mixed areas where staining was definitely positive (+ or ++), slightly positive (+) or totally negative (−). A quarter of the areas was positive in two cases (BT-7 and BT-10) and more than three-quarters in another two cases (BT-14 and M-6 RPT(rt)). Two tumors (M-9 BT-b and -c) showed heterogeneous and scattered patterns with positive areas occupying a quarter and a half, respectively. For multiple synchronous tumors, the staining patterns were similar for the separate lesions within the same cases. Only case M-6 was an exception, the renal pelvic cancer showing a heterogeneous immunoreaction, whereas the urinary bladder cancer demonstrated a diffuse distribution of positive nuclei (M-6 BT).

**Statistical analysis**

p53 abnormalities (including p53 mutations and/or overexpression) were found in 15 of 32 (47%) urothelial cancer cases. For those with multiple urothelial cancers (M-1 to -10), one case in which different tumors showed variation in the p53 status was excluded from the statistical analysis (case M-9). The highest stage tumors for each case were taken and the relationships between histopathological parameters (morphological status; whether papillary or non-papillary tumor, grade and stage) and p53 abnormalities were statistically evaluated. p53 abnormalities found in the present study were significantly related to morphological status and tumor grade (Table III). Non-papillary tumors had p53 abnormalities (8 of 10, 80%) more frequently than papillary tumors \( (P<0.05) \) and a positive relationship was found with tumor grade.

**DISCUSSION**

The present study revealed p53 abnormalities including mutations and overexpression in 15 of 32 (47%) urothelial cancer cases. In the subcategory of urinary bladder cancers, 14 of 24 (58%) cases had p53 abnormalities, this frequency being slightly higher than in previous reports.\(^3\)\textsuperscript{–}\(^6\)\textsuperscript{,}\(^18\)\textsuperscript{,}\(^19\) We demonstrated here that urinary bladder cancers are more prone to have p53 abnormalities than renal pelvic or ureteral cancers, and that changes were frequent in non-papillary and/or high grade tumors, in agreement with previous studies. Previous clinicopathological evidence has indicated a relationship between p53 mutations and a poor prognosis.\(^5\)\textsuperscript{,}\(^6\) Since p53 mutations can occur in early stages of urinary bladder carcinogenesis, it is unlikely that loss of p53 function per se determines the biological aggressiveness of the tumors, but the poorer prognosis may reflect the fact that defective p53 function facilitates accumulation of additional genetic alterations.\(^20\)\textsuperscript{–}\(^24\)

Three immunohistochemically negative cases (BT-5, -6 and M-5) were found to have 3 nonsense mutations due to b.p. deletion or insertion in the p53 cDNA, indicating that immunohistochemistry can give false-negative results. A single amino-acid change can result in a significantly longer half life than that of the wild type p53 protein. In contrast, nonsense mutations result in the production of truncated p53 proteins, whose degradation is extremely rapid, resulting in negative p53 protein accumulation.\(^5\)

A 5-b.p. insertion at the junction of exons 6 and 7 on p53 cDNA was observed in case BT-5; guanine base (third nucleotide of codon 224) was changed to adenine and the

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**Table III. Correlation between p53 Abnormalities and Histopathological Parameters in Human Urothelial Cancers**

| Morphological status (n=31)       | Total no. of cases | p53 abnormality \(^a\) | No. | %   |
|----------------------------------|--------------------|------------------------|-----|-----|
| Papillary \(^b\)                 | 21                 |                        | 6   | 29  |
| Non-papillary Grade \(n=30\)     | 10                 |                        | 8   | 80  |
| Grade 1                          | 5                  |                        | 0   | 0   |
| Grade 2                          | 15                 |                        | 4   | 27  |
| Grade 3                          | 10                 |                        | 9   | 90  |
| Stage \(n=31\)                   | 17                 |                        | 5   | 29  |
| pT1-pT4                          | 14                 |                        | 9   | 64  |

\(a\) p53 mutation and/or overexpression. 
\(b\) One case of a well differentiated adenocarcinoma (BT-15) was excluded. 
NS, no significant difference.
subsequent 5 b.p.s were identical to the initial 5 b.p.s of intron 6, suggestive of aberrant mRNA processing. Since b.p. substitution at codon 224 was within the ‘splice donor site; exon-G/GT-intron,’ mRNA processing was shifted to the next G/GT sequence of intron 6, which meets Chambon’s rule. We previously reported such aberrant splicing in mouse urinary bladder cancers using reverse transcription (RT)-PCR-single strand conformation polymorphism (SSCP) analysis. As far as we know, however, this case is the first example of a splicing mutation in a human urinary bladder cancer.

The present detailed immunohistochemical analysis demonstrated that 5 cases had heterogeneous cell populations; certain areas were immunoreactive and other areas were negative. Two tumors with heterogeneous immunoreactivity (BT-7 and BT-c of case M-9) and a single tumor with diffusely positive immunoreaction (UT-1) were negative for a p53 mutation by yeast p53 functional assay. One possible explanation is that the frozen materials used for yeast p53 functional assay might have been taken from subpopulations without p53 mutation. Alternatively, protein accumulation could be caused by a mechanism other than p53 gene mutation. The existence of intratumor genetic heterogeneity has already been reported for human urinary bladder cancers and also suggested in our rodent model. Combined use of immunohistochemistry with molecular biological techniques is useful for the detection of genetic heterogeneity in such cases.

An additional benefit of the yeast p53 functional assay is that it can estimate the allelic status of the p53 gene; if the percentage of red colonies exceeds 50%, it is strongly suggestive of loss of the wild type allele in addition to a mutation in one allele. It has been reported that the percentage of exfoliated cancer cells is small (e.g., case M-2) had already lost either p53 allele as determined by fluorescence in situ hybridization (Lee et al., unpublished data), supporting the hypothesis that loss of functional p53 might be a significant step in the development of urothelial cancers. Multifocal synchronous and metachronous development of human urothelial cancers is relatively common. Recently, several studies have suggested a common origin for multifocal urothelial cancers harboring the same genetic alterations, such as X-chromosome inactivation and loss of chromosome 9q sequences as described by Sidransky et al. or p53 mutations as reported by Habuchi et al. and Lunec et al. In contrast, data have been presented that implied the existence of independently transformed urothelial cell populations in the same patients. These findings and our animal experiments support the conclusion that multiple urothelial cancers can be of either monoclonal or multiple clonal origin. In the present study using yeast p53 functional assay, multiple synchronous tumors had identical p53 mutations and a clonal relationship was strongly suggested. One tumor (BT-b) in case M-8 might have originated from a clone retaining the wild type allele (BT-a) and the wild type allele might have been lost during the migration process.

In conclusion, we have demonstrated here that a combination approach to the assessment of p53 status (the yeast p53 functional assay plus immunohistochemistry) is a powerful tool to investigate the molecular mechanisms of human urothelial cancers. We are now trying to apply the yeast p53 functional assay to find cells with p53 mutations in urine samples of urothelial cancer patients. Even when the percentage of exfoliated cancer cells is small, this offers promise for diagnostic purposes.

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