**p53 Deletion as a Genetic Marker in Urothelial Tumor by Fluorescence in Situ Hybridization**

Hideyasu Matsuyama, Yi Pan, Ensaif A. Mahdy, Per-Uno Malmström, Anders Hedrum, Matthias Uhlen, Christer Busch, Takashi Hirano, Gert Auer, Bernhard Tribukait, Katsusuke Naito, Peter Lichter, Peter Ekman, and Ulf S. R. Bergerheim

Departments of Urology [H. M., P. E., U. B.], Pathology [T. H., G. A.], and Medical Radiobiology [Y. P., B. T.], Karolinska Hospital, 171 76 Stockholm, Sweden; Departments of Urology [P-U. M.] and Pathology [E. M., C. B.], University of Uppsala, 751 85 Uppsala, Sweden; Department of Biochemistry, Royal Institute of Technology, Stockholm, Sweden [A. H., M. U.]; Deutsches Krebsforschungszentrum, Heidelberg, Germany [P. L.]; and Department of Urology, Yamaguchi University School of Medicine, Ube, 755 Japan [K. N.]

**Abstract**

In order to investigate the significance of p53 deletion, 42 specimens of transitional cell carcinoma were analyzed by interphase cytogenetics with a fluorescence in situ hybridization technique and compared with clinicopathological and cytochemical parameters. In total, 27 (64%) and 16 (38%) specimens demonstrated p53 deletion and overexpression, respectively. The p53 deletion was significantly correlated with grade (P < 0.01), stage (P < 0.05), S-phase fraction (P < 0.05), and DNA ploidy (P < 0.01), while p53 overexpression correlated only with grade (P < 0.05). The close correlation of p53 deletion with clinicopathological parameters suggests p53 deletion to be of clinical importance to indicate the malignant potential of human urothelial tumors.

**Introduction**

Identification of p53 nuclear overexpression by immunohistochemical assays has been reported to correlate well with p53 mutations, as determined by direct sequencing in a variety of tumor types (1–3). However, recent analysis suggests that the relationship between p53 gene mutation and overexpression of mutant protein is not clear-cut and that mutations are not directly reflected in p53 overexpression as assessed by immunohistochemistry (4). p53 mutations have been identified as being closely linked to chromosome 17p allele losses in most common tumor types including bladder cancer (5, 6). It is still controversial, however, whether the point mutations in the p53 gene and the production of missense p53 protein occur before the loss of the wild-type allele (7). Recent studies indicate that the p53 mutation may precede loss of one p53 allele in colorectal and several tumors (8), whereas Nakai et al. (9) demonstrated that loss of a 17p may precede p53 mutation in chronic myelogenous leukemia.

The FISH technique permits rapid characterization of genetic aberrations in interphase nuclei of individual cells in small volumes of histological and/or cytological samples (10–12). 17p deletion studies using the FISH technique have been reported in breast cancer (11), but to our knowledge, this is the first report showing the relationship between 17p deletion detected by the FISH technique and p53 overexpression in urothelial tumors.

The aim of this study was to clarify the clinical significance of chromosome 17p deletion, in particular deletion of the chromosomal region containing p53 locus, and compare it with p53 overexpression as well as with direct sequencing of the selected cases in urothelial tumor. In addition, the possibility of precedence of 17p deletion compared with p53 mutation will be discussed.

**Materials and Methods**

**Patient Materials.** Forty-two specimens from 41 patients with transitional cell carcinoma (39 bladder cancers and 2 cancers of the renal pelvis) and 8 specimens of histologically normal bladder mucosa were studied. The average age of the patients was 70 years ranging from 30 to 86 years. The tumor samples were graded according to the WHO classification system with modification (13, 14) and classified according to tumor-nodes-metastasis classification (15). These specimens were collected by cold cup biopsy, transurethral resection of the bladder tumor, total cystectomy, urine with positive cytology, and radical nephroureterectomy from 38, 3, 2, 1, and 2 patients, respectively. All specimens were prepared by the touch biopsy technique as reported previously (12).

The parallel slide preparation was stained with May-Grünwald-Giemsa staining and reviewed by the cytologist (G. A.) to assess the quality and quantity of the tumor sample. The slides were stored at −70°C until use.

**Immunohistochemistry.** The touch biopsy specimens were used for immunohistochemical studies. The rabbit polyclonal anti-p53 antibody (NCL-p53-CM1; Novoceastra Laboratories, Newcastle, United Kingdom) was used as the antibody against mutant p53 protein at the dilution of 1:700, and overnight incubation was done at 37°C in a moisture chamber. The detection was carried out with biotinylated anti-rabbit immunoglobulin (1:200 dilution; Vector, Burlingame, CA), followed by incubation with peroxidase-conjugated streptavidin (Vecta Elite kit; Vector) The 3,3'-diaminobenzidine tetrahydrochloride substrate (0.06%; Sigma) was used for coloration. The established breast cancer cell lines (MDA231 and MCF7, provided by American Type Culture Collection) were used as positive and negative controls, respectively. The specimen was examined by three independent observers (H. M., U. B., and G. A.), and judged as positive for immunostaining when the specific brown color was accumulated in nuclei of monolayered cells as compared with the surrounding cytoplasm.

**DNA Direct Sequencing with PCR.** Genomic DNA was extracted after proteinase K digestion from the frozen section or paraffin-embedded samples by using microdissection methods. Two pairs of oligonucleotide primers (one pair for outer amplification and another primer labeled with biotin for inner amplification) were designed for exon 5, 6, 7, and 8 based on the primer sequence data published previously (16). Amplification was performed in a 50-μl volume containing 5 μl of DNA sample (cell lysate), 10 μM Tris-HCl, 50 mM KCl, 0.1% Tween 20, 1.5 mM MgCl2, 0.1 mM concentrations each of deoxynucleotide triphosphates, 10 pmol each of primer, and 1 unit of Taq polymerase. As outer amplification, 35 cycles of amplifications were carried out in an automatic thermal cycler (Perkin-Elmer, Norwalk, CT) at 94°C, 55°C, and 72°C for 30, 30, and 60 s except for the last cycle of extension (72°C) for 10 min. Subsequently, 30 cycles of inner amplification was performed by using 5 μl of outer amplification products at the same condition except for the annealing temperature at 65°C. Mutations of the p53 gene were analyzed by the solid-phase DNA sequencing using magnetic beads (Dynabead M280 Streptavidin; Dynal AS, Oslo, Norway), and automated laser fluorescent sequencer (Pharmacia, Biotech, Uppsala, Sweden).
Fluorescence in Situ Hybridization. The biotin-labeled chromosome 17 centromeric DNA probe (D17Z1; Oncor, Gaithersburg, MD) and 3 cosmid DNA probes covering the p53 locus (17p13.1), which was kindly provided by Dr. A. Poustka, were used in this study. The cosmid probe was labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) using nick-translation. The FISH technique was performed as described elsewhere (10, 12). In brief, the specimens were denatured at 70°C for 2 min in a denaturation solution [70% formamide-2 × SSC (standard saline citrate), 1 × 0.15M sodium chloride, 0.15M sodium citrate], pH 7. Forty ng of cosmid DNA were ethanol precipitated with 8 μg of salmon sperm DNA and 1 μg of human placental DNA (Cot-i DNA; Gibco BRL, Gaithersburg, MD), dissolved in 5 μl of hybridization mixture (50% formamide, 10% dextran sulfate, and 2 × SSC), denatured at 75°C for 5 min, preannealed at 37°C for 10 min, and applied onto the denatured specimens. Fifteen ng of centromeric probe were prepared in the same way as described above but without human placental DNA addition and preannealing. The hybridization was done at 37°C overnight in a moist chamber. Posthybridization washings were done in 50% formamide-2 × SSC, pH 7, at 42°C for 5 min (4 washings) and in 0.1 × SSC at 60°C for 5 min (3 washings). FITC-conjugated avidin (Sigma) and anti-digoxigenin rhodamine (Boehringer Mannheim) were used as detecting materials for centromeric and cosmid probes, respectively. After addition of fluorescent materials, the slides were washed in 4 × SSC-0.1% Tween 20 at 42°C for 5 min (3 washings) and counterstained with 4,6-diamidino-2-phenylindole dihydrochloride.

Evaluation of the Signals. Two observers (H. M. and Y. P.) performed independent evaluation of the FISH slides in a blinded manner. More than 150 nuclei (151–202; mean, 165) were analyzed from each specimen using an epifluorescence microscope (Nikon, Tokyo, Japan) through a triple band pass filter for 4,6-diamidino-2-phenylindole dihydrochloride/FITC/trichloroacetic acid (Chroma Technology, Brattleboro, VT). Chromosomal numerical aberrations were diagnosed as chromosomal loss (monosomy) or gain (trisomy or tetrasomy) when the percentage of nuclei with one, three, or four signals exceeded 10%, respectively. Aneusomy was defined when two or more different types of numerical aberrations were observed in a sample. Deletion was defined as when the fraction with decreased number for cosmid signals compared with centromeric signals including monosomy fraction (decreased fraction) exceeded 30% of all nuclei counted. The cutoff level was set to 30% based on the upper limit (mean + 2 SD) of the decreased fraction of the p53 deletion.
normal bladder mucosa in a percentage of 29.9%. If the fraction in which no spot was identified for cosmid probes (zero spot fraction) exceeded more than 20% of all nuclei counted, the sample was excluded from the study. To prove the statistical differences and correlations, ANOVA and a contingency table with Fisher's exact test were performed by using StatView (Abacus Concepts, Inc., Berkeley, CA).

**Results**

**Overview of the Study.** The hybridization efficiency of the two probes were determined by the two signal fraction in normal lymphocytes. The hybridization efficiency of the p53 cosmid probe and D17Z1 was 89 ± 3% (SD) and 98 ± 1%, respectively. Forty-two specimens were analyzed by FISH and by the immunohistochemical staining for anti-p53 antibody. The direct DNA sequencing was performed on 10 specimens. The decreased cosmid signal fraction (decreased fraction) in histologically normal bladder mucosa was 23.3 ± 3.3%. The average zero signal fraction of clinical specimens was 4.8 ± 3.3%. The interindividual variation regarding the decreased fraction by two independent observers was 6.4 ± 5.5%. Monosomy, disomy, trisomy, and aneusomy of chromosome 17 were detected in 8, 8, 11, and 15 specimens, respectively. Four of the 8 specimens with disomy demonstrated p53 deletion, whereas 14 of the 15 specimens with aneusomy showed p53 deletion.

In total, 27 cases (64%) demonstrated p53 deletions, while only 16 (38%) showed p53 overexpressions. The point mutations were detected in 4 of the 10 specimens. Out of the 27 specimens with p53 deletions, 16 (59%) showed p53 overexpression. None of the 15 cases lacking p53 deletion demonstrated p53 overexpression. The frequency of p53 overexpression in tumors with p53 deletion was significantly higher (59%, 16/27) than the frequency in tumors without the p53 deletion (0%, 0/15). A significant correlation was found between p53 deletion and p53 overexpression. Eleven of the 27 (41%) cases with p53 deletion showed no p53 overexpression. Of these cases, 8 were high grade and/or high stage. Concerning point mutations by direct sequencing, all 4 specimens with point mutations demonstrated not only p53 overexpression but also p53 deletion; whereas of the 6 specimens without point mutations, p53 overexpression and p53 deletion were found in 3 and 6 specimens, respectively (Table 1).

**p53 Alteration in Relation to Clinical Parameters.** Table 2 summarizes the relationship between p53 alteration (p53 deletion and p53 overexpression) and several clinicopathological parameters. The p53 deletion was significantly correlated with grade (33% in low grade versus 87% in high grade tumor), stage (50% in superficial versus 92% in invasive cancer), and DNA ploidy (35% in diploid versus 75% in aneuploid cancer), while p53 overexpression correlated only with grade (17% in low grade versus 54% in high grade tumor). When the p53 decreased fraction was compared with histopathological grade, a significant difference was seen between Grades Ila and IIB (Fig. 1). A significant correlation was found between p53 decreased fraction and the S-phase fraction calculated from a flow cytometric DNA histogram (r = 0.621, P < 0.01).

**Discussion**

Recent reports have shown a close relationship between nuclear overexpression of p53 protein and tumor progression in bladder cancer (3). The frequency of p53 overexpression is varying and ranges from 20 to 50% (3, 17). Our results (38%) are well in agreement with previous reports. In our study, 58% of the invasive tumors demonstrated p53 overexpression, a figure almost identical to the data by Sidransky et al. (6). Previous cytogenetic and RFLP studies have revealed that 17p deletion is highly associated with p53 mutation. Our data clearly demonstrate the close correlation between p53 deletion and clinicopathological features (grade, stage, DNA ploidy) which are regarded as the most important prognostic factors in bladder cancer, whereas p53 overexpression correlated only to grade with lower significance (P < 0.05). The S-phase fraction has also been reported as one of the most important prognostic factors reflecting patient prognosis, as well as the growth fraction of the tumor cell (18), whereas little is known about the relation of the S-phase fraction to p53 mutations (19). The wild type p53 protein arrests the cell cycle at G1 by regulating the transcription factors, presumably resulting in the decrease of S-phase fraction (7). The increase of S-phase fraction is likely to occur in the tumor cell if the wild type p53 protein is lost. The striking correlation between the S-phase fraction and the p53 deletion could coincide with this assumption, suggesting the p53 deletion to reflect the loss of tumor suppressor function of the p53 gene.

Even though p53 overexpression is frequently accompanied by the p53 deletion, 11 (41%) specimens with p53 deletion showed no p53 overexpression. There may be two possible explanations for this discrepancy. One is that the mutation occurred in the region where it cannot be detected in epitopes studied by immunohistochemical staining. Two of these discrepant specimens, however, showed no point mutation by direct DNA sequencing, although the examined exons were limited, ranging from 5 to 8. Another is that 17p (p53) deletion may precede the p53 mutation. The p53 deletion could be detected in 6 specimens despite the observation that these showed no mutation by direct sequencing. The result supports the hypothesis that 17p deletion affects the expression of p53 gene.

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**Table 2** Association between p53 alterations and clinicopathological parameters

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>Cases with p53 deletion/none with each category (%)</th>
<th>Cases with p53 overexpression/none with each category (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade (≤Ila)</td>
<td>6/18 (33)a</td>
<td>3/18 (17)b</td>
</tr>
<tr>
<td>High grade (≥Ilb)</td>
<td>21/24 (87)a</td>
<td>13/24 (54)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial (≥T1)</td>
<td>14/28 (50)</td>
<td>7/28 (25)</td>
</tr>
<tr>
<td>Invasive (≥T2)</td>
<td>11/12 (92)</td>
<td>7/12 (58)</td>
</tr>
<tr>
<td>DNA ploidy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid</td>
<td>5/14 (36)</td>
<td>3/14 (21)</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>19/23 (83)</td>
<td>11/23 (48)</td>
</tr>
</tbody>
</table>

a P < 0.01 (Fisher’s exact test).

b P < 0.05 (Fisher’s exact test).

cTwo cases with Tis (carcinoma in situ) were excluded.

dFive cases were excluded because examination was not done.
may precede the p53 mutation as has been indicated previously in chronic myelogenous leukemia (9). If this hypothesis holds true, it should be of clinical value in the detection of the 17p deletions because one might identify the cases which might be at risk for tumor progression. We do not have any convincing evidence whether p53 deletion will predict the patient’s prognosis, since specimens used in this study were collected recently and only a couple of cases had recurred. In one case (tissue sample no. 11 and no. 12), however, the pattern of p53 overexpression changed from negative to positive in the second specimen (case 12) taken 4 months later in spite of the consistency of the p53 deletion pattern in the decreased fractions of 56 and 54% (Table 1). Interestingly, the patient had tumor progression (Grade IIa to IIb) 9 months after the first specimen. This case suggests that p53 deletions closely correlate with the malignant potential more than the p53 overexpression and hereby may represent a genetic marker of urothelial tumors (20). However, since p53 mutations still occur in a small fraction of the tumors which have retained both parental 17p alleles, p53 immunohistochemical studies will also be of value.

In conclusion, this study presented (a) the significance of 17p (p53) deletion in urothelial tumor and (b) the implication of the precedence of chromosome 17p deletion compared with p53 overexpression.

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References

hybridization

in situ hybridization

p53 deletion as a genetic marker in urothelial tumor by fluorescence in situ hybridization

Hideyasu Matsuyama, Yi Pan, Ensaf A. Mahdy, et al.


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