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Physical Deletion of the p53 Gene in Bladder Cancer

Detection by Fluorescence in Situ *Hybridization*

Guido Sauter,* Guoren Deng,† Holger Moch,‡ Russell Kerschmann,* Kouji Matsumura,* Sandy De Vries,* Tracy George,* Jose Fuentes,* Peter Carroll,* Michael J. Mihatsch,‡ and Frederic M. Waldman*

From the Division of Molecular Cytometry, Department of Laboratory Medicine, University of California, San Francisco, California; California Pacific Medical Center Research Institute,† San Francisco, California; and Department of Pathology,‡ University of Basel, Basel, Switzerland*

To understand better the role of physical p53 deletion in bladder cancer, 106 formalin-fixed and 45 unfixed bladder tumors were examined using fluorescence* in situ *hybridization. Probes for centromere 17 and the p53 locus were hybridized simultaneously to interphase tumor cells to analyze p53 and chromosome 17 copy number on a cell by cell basis. 17p deletion was found in four of 43 pTa tumors, 18 of 43 pT1 tumors and 29 of 58 pT2-4 tumors (P = 0.0001). 17p deletion was also highly correlated with grade (P = 0.0001) and with p53 immunostaining (P = 0.0005). Chromosome 17 polysomy was associated with stage, grade, 17p deletions, and p53 immunostaining (P = 0.0001). The strong difference in centromere 17 copy number and 17p deletions between pTa and pT1 tumors supports a relevant biological distinction between pTa and pT1 tumors. (Am J Pathol 1994, 144:756–766)

p53 is a tumor suppressor gene located on chromosome 17p13.1.[1] It codes for a DNA binding protein and is apparently involved in transcriptional regulation.[2,3] Transfection of wild type p53 genes into human tumor cell lines has been shown to arrest cell growth in the G1 phase of the cell cycle.[4] Most recently it was suggested that the biological function of p53 is as a cell cycle checkpoint, assuring the appropriate internal environment for cell replication and division.[5] Such a key role for the p53 gene product would explain why p53 mutations appear in almost every kind of human tumor.[6]

Missense p53 mutations frequently result in a prolonged half life of the protein[7] and thus are associated with increased levels detected immunohisto chemically.[8–11] p53 protein overexpression as well as the presence of p53 mutations, have been shown to have clinicopathological correlations in a variety of tumors.[9,12–19] In bladder cancer, p53 protein expression correlates with tumor grade and stage[20,21] as well as with progression in pT1 tumors.[22]

It is hypothesized that tumor suppressor genes are generally inactivated by mutation of one allele and deletion of the second.[23] Using restriction fragment length polymorphism (RFLP) analysis and immunohistochemistry or DNA sequencing together, a correlation between 17p deletions and p53 protein alterations has been shown in tumors of several organs including bladder.[21] However, other studies have failed to show a clear association between p53 protein expression[24,25] or presence of p53 mutations[24,26–28] and loss of heterozygosity (LOH) at 17p13. The relationship between 17p deletion and p53 mutation is further confounded by evidence for dominant acting p53 mutations[29,30] as well as a recently proposed second tumor suppressor gene on 17pter.[31–33]

Fluorescence *in situ* hybridization (FISH) is a powerful tool to visualize quantitative genomic alterations on a cell by cell basis.[34,35] Using a dual labeling technique with probes for the centromeric region of

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Address reprint requests to Dr. Guido Sauter, University of Basel, Department of Pathology, Schoenbeinstrasse 40, 4003 Basel, Switzerland.

chromosome 17 and for the p53 gene locus simultaneously, we recently examined 17p deletions in fresh and formalin-fixed breast cancer tissue and found a strong correlation between FISH and RFLP analyses.[35]

Using FISH, we have addressed the following questions in bladder tumors: 1) How frequent are physical 17p deletions in bladder cancers of different grade and stage? 2) To what extent do physical 17p deletions include both the p53 locus and the 17p terminus? 3) What is the relationship between 17p deletions and p53 immunostaining? and 4) What is the relationship of tumor proliferation to 17p deletion?

Materials and Methods

Patient Material

Tumor material consisted of imprint preparations from 45 cases and dissociated cells from 106 formalin-fixed, paraffin-embedded specimens. Imprint preparations were made immediately after surgery from unfixed bladder tumor tissue and stored in nitrogen at −20 C. Formalin-fixed tissue samples were randomly chosen from the archives of the Department of Pathology, University of Basel, and dissociated as described below. Combined samples were from 137 primary tumors and 14 superficial recurrences. Forty-three tumors were confined to the bladder mucosa (pTa), 43 showed invasion of the lamina propria (pT1), and 58 were muscle-invasive (pT2-T4). Forty-four tumors were classified as grade 1, 51 were grade 2, and 51 were grade 3. Inadequate biopsy size or mechanical damage prevented appropriate grading (and staging) in five[7] tumors. Bromodeoxyuridine (BrdUrd) labeling was done in 34 of the fresh tumor samples as previously described.[36] RFLP allelotyping was performed in 14 of the fresh tumors. p53 immunostaining was done in all 106 formalin-fixed tumors.

Histology and Immunohistochemistry

Four-μ sections were used for hematoxylin and eosin and immunostaining. Tumor stage was defined according to Union Internationale Contre le Cancer.[37] Because of the limitations of transurethral biopsies in accurately determining the depth of invasion of higher stage bladder cancer, all tumors showing muscle invasion were categorized into one group (pT2-4). Tumor grading was according to World Health Organization,[38] using grades 1 to 3 with pure papillomas (grade 0) excluded. For visualization of BrdUrd incorporation the monoclonal antibody IU4 was used as previously described.[36] BrdUrd Labeling Index (BrdUrd LI), defined as the percentage of BrdUrd labeled tumor cells, was determined by scoring at least 2,000 tumor cells in 10 well-labeled high-power fields. Formalin-fixed tissue sections were stained for p53 expression using the polyclonal antibody CM1 (1:4,000; Medac GmbH, Hamburg, Germany), recognizing mutant and wild type p53 protein, and standard indirect immunoperoxidase procedures.[39] Breast tumors with known overexpression of p53 were used as positive controls. Tumors showing distinct nuclear staining in at least 10% of tumor cells were considered positive for p53 overexpression to minimize the risk of false positivity.

Cell Dissociation

Fifty-μ sections of formalin-fixed, paraffin-embedded tumor tissue were used. The tissue block with the highest proportion of tumor cells (at least 60%) was selected, and blocks containing large numbers of histiocytes (as in granulation tissue) were avoided. Sections were incubated at 55 C in 3 ml of xylene for 1 hour, and then again overnight. Sections were rehydrated in ethanol (100%, 95%, 80%, 50%) for 1 hour each and incubated in 50% ethanol for 60 hours at 4 C. Sections were then washed in distilled water for 1 hour, treated with 2 ml 0.5% pepsin (pH 1.5) at 37 C for 1 hour, and vortexed vigorously. Suspended nuclei in the supernatant were removed, and pepsin digestion was stopped with 2 ml fetal calf serum. Nuclei were pelleted at 180*g* for 5 minutes and washed twice in phosphate-buffered saline. Cells were resuspended in 2 ml 50% ethanol. Cells then were washed in Carnoy's fixative (methanol/glacial acetic acid, 3:1) and dropped onto cleaned glass slides. Slides were stored at −20 C for up to 6 months.

DNA Probes and Probe Labeling

A probe for the chromosome 17 repetitive pericentromeric sequence and a second probe for the 17p locus of interest were used simultaneously. The following probes were used: centromere 17 (p17H8), p53 (a combination of three contiguous cosmid probes at the p53 locus at 17p13, courtesy of Dr. Ray White, Howard Hughes Medical Institute, Salt Lake City, UT), and C144 (a cosmid probe at the 17p telomere). Using standard nick translation protocols cosmid DNA was labeled with digoxigenin-11-dUTP and centromere DNA was labeled with biotin-14-dATP.

Fluorescence in Situ *Hybridization*

All slides were fixed in methanol/acetic acid (3:1) and subsequently air-dried. FISH was performed as previously described[34,35] with modifications. Cells on slides were denatured in 70% formamide/2X standard saline citrate (SSC) (1X SSC is 0.15 mol/L NaCl, 0.015 mol/L NaCitrate), pH 7, at 70 to 75 C for 2.5 minutes. After dehydration in graded ethanols, samples were treated with proteinase K (Sigma). Touch preps were treated at 0.5 μg/ml, whereas dissociated cells received 2.0 μg/ml in phosphate-buffered saline (pH 7.0) for 7 minutes at 37 C, followed by ethanol dehydration. The hybridization mixture was denatured for 5 minutes at 75 C, allowed to reanneal for 30 minutes at 37 C, and applied to denatured cells on slides. Ten μl of hybridization mixture consisted of 10 ng cosmid probe, 4 ng centromeric probe (20 ng for formalin-fixed cells) as well as 10 ng unlabeled, sonicated (200 to 500 bp) human placental DNA (Sigma Chemical Co., St. Louis, MO) in 50% formamide, 10% dextran sulphate and 2X SSC (pH 7). Hybridization was overnight at 37 C. Lymphocyte controls were used to assure probe specificity. Slides were washed three times in 50% formamide/2X SSC (pH 7) and twice in 2X SSC for 10 minutes each at 45 C. After another wash in 2X SSC at room temperature and preblocking in 1% bovine serum albumin/4X SSC for 5 minutes, slides were immunostained in three steps (five steps for formalin-fixed cells) at room temperature for 60 minutes each. The first staining consisted of 13 μg/ml fluorescein conjugated sheep anti-digoxigenin (Boehringer Mannheim, Indianapolis, IN), diluted in 1% bovine serum albumin/4X SSC. The slides were then washed in 4X SSC, 4X SSC/0.1% Triton X-100, 4X SSC and PN-Buffer (0.1 mol/L NaH_2PO_4, pH 8, and 0.1% Nonidet P-40) for 10 minutes each and preblocked with PNM (5% Carnation dry milk, 0.02% Na-azide in PN buffer) for 5 minutes. The second antibody incubation consisted of 0.3 mg/ml fluorescein isothiocyanate-conjugated anti-sheep IgG (Sigma), diluted in PNM, followed by three PN washes for 10 minutes each. After another PNM block for 5 minutes, the third immunochemical staining was done, using 3 μg/ml Texas Red avidin (Vector Laboratories, Burlingame, CA), diluted in PNM. After three PN washes for 10 minutes each, nuclei were counterstained with 0.07 μg/ml 4.5-diamino-2-phenyl-Indole (DAPI) in antifade solution. Amplification of Texas Red signals was performed before DAPI staining for dissociated cells from paraffin blocks. Slides were incubated with 5 μg/ml biotinylated anti-avidin (Vector), followed by three PN washes (10 minutes) and then another layer of 3 μg/ml Texas Red avidin (Vector) diluted in PNM. If tumor cell signals were weak, presumably due to a low hybridization efficiency, hybridization was repeated using the same protocol with an increased proteinase K concentration (up to 15 μg/ml). Proteinase K concentration was reduced if excessive nuclear damage was observed.

Scoring of FISH Signals

Cells were selected for scoring according to morphological criteria using DAPI staining. For imprint preparations, cells in groups or sheets were selected because these were considered to be epithelial in origin. For dissociated cells, small round, lymphocyte-like cells were disregarded. Copy numbers of centromere 17 and 17p signals were counted for 50 to 100 nuclei. Because the optimum proteinase K concentration varied greatly between cases, the use of an external control for hybridization efficiency was not possible. Therefore, cells were only scored when at least one bright cosmid signal and one bright centromere signal were present, to avoid misinterpretation due to insufficient hybridization efficiency. Slides were only analyzed if the majority of cells were interpretable in representative areas. Two signals were counted as one signal if they were situated very close to each other (0.5 μm) to avoid misinterpretation due to sister chromatids of cells in S- or G2M-phase.

As a measure of deletion, the percentage of cells containing either one copy of centromere 17, or fewer cosmid signals than centromeric signals, was calculated for each hybridization (defined as the percentage of deletion). In each case, the average copy number of centromere 17 was also calculated. Polysomy of chromosome 17 was arbitrarily defined as an average centromere 17 copy number ≥ 2.3.

Allelic Loss

Allelic loss was determined by polymerase chain reaction (PCR) amplification of DNA extracted from frozen tumor tissue by standard protocols.[40] The following forward and reverse primers (1 and 2) were used for amplifying fragments covering polymorphism sites detected by restriction enzyme digestion, as noted.

5.1: CAATGGATGATTTGATGCTG (Exon 4, BstUI, 196bp)
5.2: TGGTAGGTTTTCTGGGAAGG
6.1: AGGTCTGGTTTGCAACTGGG (Intron 6, MspI, 107bp)
6.2: GAGGTCAAATAAGCAGCAGG

8.1: TCAGAAGGAAGTAGGAAGGACTCAG (3′flank, BamHI, 90bp)
8.2: GAAGAGCCTCGGTTATGGGTATACA

One hundred ng of DNA from tumor and normal tissue were amplified by PCR. The annealing temperature in each cycle was 60 C. Five μl of PCR product was digested with 10 units of restriction enzyme for 2 hours. The digested fragments were separated on a 3% agarose gel or 6% polyacrylamide gel. DNA bands were visualized either by ^{32}P labeling (using 5′-end-labeled primer in PCR) or by ethidium bromide staining. LOH was defined as a relative loss in allelic band intensity of tumor as compared to normal DNA. Losses were equivalent to 50% relative band intensity reduction as defined by densitometry.

Statistical Analysis

Correlation of 17p deletions or aneuploidy with p53 immunostaining, tumor stage, and grade was assessed by contingency table analysis. Correlation of 17p deletions, grade and stage with BrdUrd LI was examined using a U-test. Other analyses were as described.

Results

FISH Analysis

FISH with a gene-specific cosmid probe for p53 and a centromere repeat probe for chromosome 17 was used to determine p53 deletions. Whereas centromere signals were bright and clear in most of the nuclei, cosmid signal intensity was less stable presumably due to variable hybridization efficiency. Cosmid signals were weaker in areas with high cell density, in cells rich with cytoplasm, and in slides stored for longer than 1 year in single cells in lower density or in recently prepared slides. Increased proteinase K treatment tended to improve (sometimes dramatically) cosmid signal intensity at the cost of some cell damage, especially of single cells. Bright centromere and cosmid signals in the majority of cells was required for analysis of a given slide. Hybridization was successful in 151 tumors. Hybridization was not successful in 24 additional formalin-fixed and five fresh cases because cosmid signals were too weak (14 cases), background staining was too high,[4] there was cell damage even without proteinase treatment,[5] there was too low a cell yield after dissociation,[5] or no identifiable epithelial cells were present in the imprint preparation.[1] The average number of scored cells was 106 (range 33 to 256) in imprint preparations and 119 (71 to 155) in formalin-fixed tumors.

The percentages of p53 deletion for all cases are shown in Figure 1. A small fraction of cells with fewer p53 signals than centromere 17 signals was always present, presumably due to incomplete hybridization of cosmid probes. A cutoff level of 40% of deletion was selected to define deletion. This was based on the finding that only six of 151 cases showed a percentage of deletion between 30 and 50%. A constant finding of a homogeneous centromere 17/p53 signal pattern in groups of cells was additionally required in imprint preparations, to confirm clonality of a lesion. This was found in four of five imprint preparations with a midrange percentage of deletion (40 to 70% deletion). In these cases, there were groups of deleted as well as of nondeleted epithelial cells, suggesting subpopulations with 17p deletion. Another tumor showed a heterogeneous population with eight to 10 centromeres 17 and seven to nine p53 signals. In the absence of a clonal arrangement of cells with less p53 than centromere 17 signals, this case was not considered to be deleted, and the increased fraction of cells with deletion (58%) was attributed to inefficient hybridization. The 17 centromere/p53 signal distribution for the largest cell subpopulation with deletion for each case is shown in Table 1. It is interesting to note that the predominant population in cases with 17p deletion showed centromere copy numbers greater than two in the majority of cases (36 of 50). A fluorescence photomicrograph of a representative case with three centromeres 17 and two p53 signals is shown in Figure 2.

To define the size of chromosomal deletions, a subset of 41 tumors was examined with probes for both p53 and for C144 (located distally to p53 at 17pter). No deletion of either probe was found in 22 cases, and deletion for both probes was seen in 18 cases. Only in one case was there a discrepancy between C144 and p53: in that case 74% of cells had a deletion for p53, and only 15% for C144, consistent with an interstitial deletion including the p53 locus. No tumors showed deletion of C144 and not p53.

Relationship between 17p Deletion by FISH and LOH

Agreement between FISH detection of p53 deletion and LOH was seen in 11 of 14 tumors (Figure 3). In 1 of the discrepant cases a clear deletion was found by FISH (two centromeres/one p53 signal, 90% cells with deletion), yet there was no allelic loss (10% LOH) by RFLP analysis. Two other cases showed LOH by

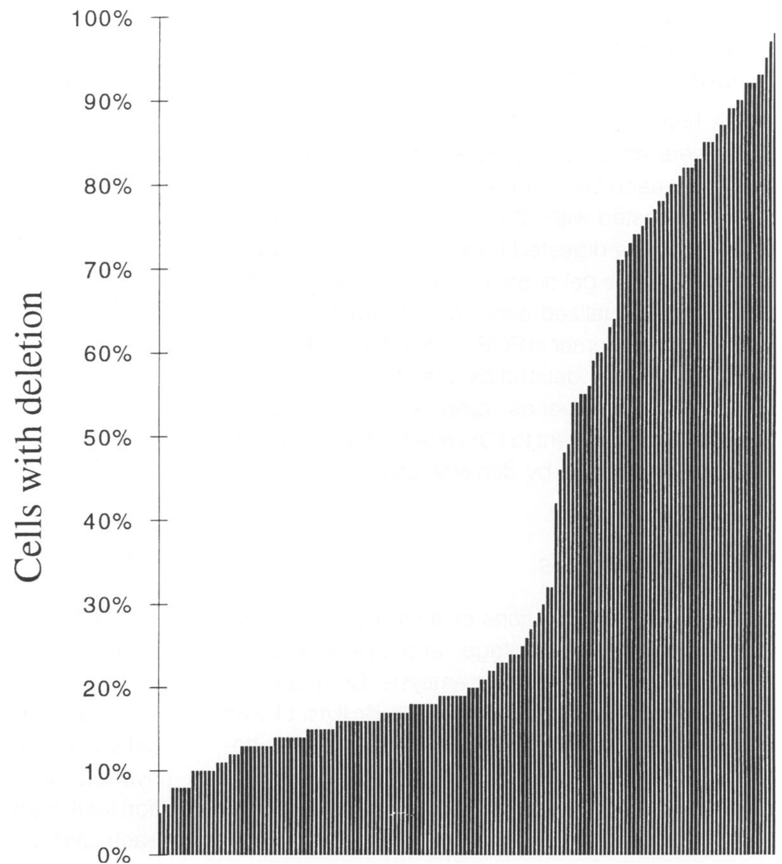

Figure 1. *Fraction of tumor cells with p53 deletion by FISH. Each bar represents one case. Cells with p53 deletion were defined as having fewer p53 signals than chromosome 17 centromeric signals or single p53 and centromere 17 signals.*

Table 1. *Patterns of Chromosome 17 and p53 Copy Number*

	Centromere/p53*	N (%)
Deletion	2/1	14 (26%)
	3/2	12 (22%)
	4/2	16 (30%)
	4/3	5 (9%)
	Other†	7 (13%)
No deletion	2/2	75 (77%)
	3/3	7 (7%)
	4/4	13 (14%)
	Other‡	2 (2%)

* Copy number of chromosome 17 centromere (numerator) and p53 (denominator) in the predominant population of tumor nuclei.
† Other deletions: 1/1, 4/1, 6/3 (2), 6/4 (2), 8/4.
‡ Other nondeletions: 5/5, 9/8 (considered no deletion because of somewhat lower hybridization efficiency for cosmid probes than for centromere probes).

RFLP analysis (LOH was 60% and 90%) but did not show deletion by FISH. Both of these tumors had three centromeres and three p53 signals in the majority of cells.

Association with Tumor Grade, Stage, and p53 Immunostaining

The relationship between 17p deletion, chromosome 17 copy number, tumor grade and stage, and p53 immunostaining is shown in Table 2. There was a strong correlation between the presence of 17p deletions and tumor stage ($P < 0.0001$) or grade ($P < 0.0001$). This was particularly driven by a sharp difference between pTa and pT1 tumors ($P = 0.0004$) and between grade 1 and grade 2 tumors ($P = 0.0002$). In contrast, there was no significant difference in the prevalence of p53 deletion between pT1 and pT2-4 tumors ($P = 0.48$) or between grade 2 and grade 3 tumors ($P = 0.36$).

The average centromere 17 copy number showed a strong correlation with tumor grade and stage ($P < 0.0001$; Kruskall-Wallis analysis). This relationship was especially clear if a cutoff value of 2.3 centromere 17 signals per cell was used to define polysomy. Differences in tumor polysomy were most prominent between pTa and pT1 tumors ($P < 0.0001$) or grade 1 and grade 2 tumors ($P < 0.0001$) and were less clear between grade 2 and grade 3 tumors ($P = 0.0157$) or between pT1 and pT2-4 tumors ($P = 0.074$). There was also a strong association between chromosome 17 polysomy and 17p deletions ($P < 0.0001$).

Positive p53 immunostaining was associated with chromosome 17 polysomy ($P < 0.0001$) or the presence of 17p deletions, if all tumors were included (P =

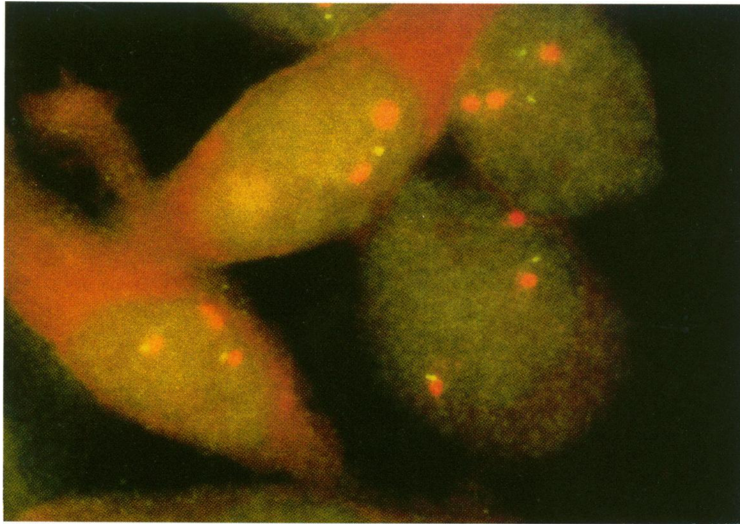

Figure 2. *FISH for p53. Cells were hybridized with probes for chromosome 17 centromere (red) and p53 gene (green). Three cells have three centromeric and two p53 signals, whereas one cell shows two centromeric and one p53 signals. Magnification 1,000×.*

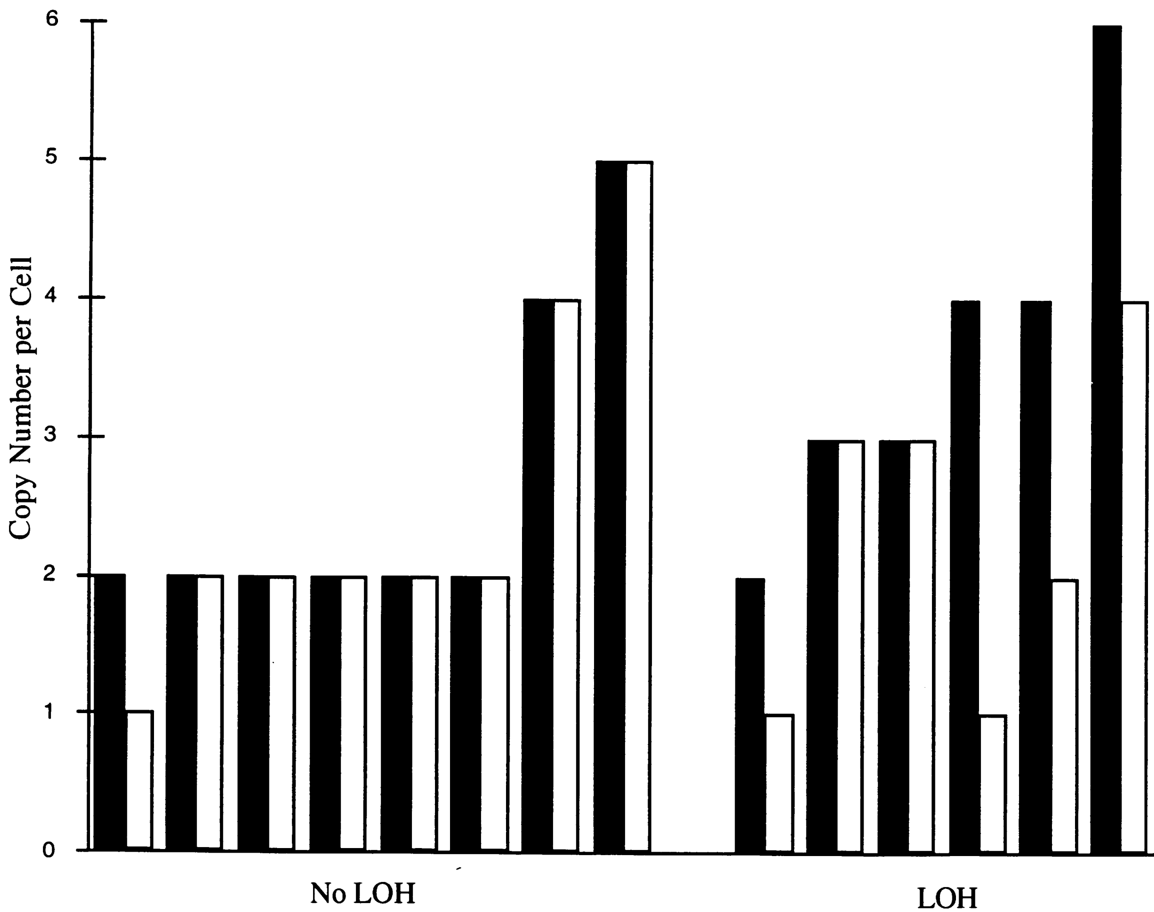

Figure 3. *Comparison of FISH and LOH analyses. Results are shown for 14 tumors, grouped by the presence of LOH. For each case, the black bar signifies chromosome 17 copy number and the white bar p53 signal copy number (in the predominant tumor cell population).*

0.0005). An association between p53 immunostaining and 17p deletion was retained within the subset of 25 pT1 tumors (17p deletions found in seven of 10 p53 positive but only in two of 15 p53-negative tumors; P = 0.0038) but was not seen in the 40 pT2-4 tumors (17p deletions found in 13 of 27 p53-positive

Table 2. *Association of 17p Deletions and Chromosome 17 Aneusomy with Tumor Stage, Grade, and p53 Immunostaining*

		Stage			Grade			p53		17p	
		pTa	pT1	pT2–4	1	2	3	Neg	Pos†	No deletion	Deletion
17p*	No deletion	39	25	29	40	30	24	50	24		
	Deletion	4	18	29§	4	21	27§	10	22‖		
av c17*‡	<2.3	39	19	15	39	22	11	42	18	63	13
	≥2.3	4	24	43§	5	29	40§	13	33§	36	40§

* Numbers listed are the number of tumors in each category.
† Positive: >10% of tumor cells positive.
‡ Average centromere 17 copy number per cell.
§ $P < 0.0001$ (contingency table analysis).
‖ $P = 0.0005$ (contingency table analysis).

and in seven of 13 p53-negative tumors; P = 0.74). p53 immunostaining of a representative case is shown in Figure 4.

BrdUrd LI

In vitro labeling with BrdUrd was done in 34 of the unfixed tumor specimens. In this small subset no significant difference was found between the BrdUrd LI of 17 tumors with p53 deletion by FISH (15.5% ± 6.1) and 17 tumors without deletion (19.2% ± 11.0, P = 0.199). There was also no association between chromosome 17 polysomy and BrdUrd LI (disomic: 16.2 +/− 10.8; polysomic: 18.0+/− 7.1; P = 0.676).

Discussion

FISH analysis was used to detect deletion of p53 in 151 bladder tumors. A strong association between the presence of p53 deletions and tumor grade and stage was seen. There was a striking increase in prevalence of p53 deletion in stage pT1 tumors compared to noninvasive pTa tumors.

Because FISH can be applied to formalin-fixed tissues, we were able to examine a patient set in which all grades and stages of bladder cancer were well represented. Our data confirm a high frequency of 17p deletions in high-grade and high-stage tumors as previously reported.[21,42–46] The low frequency of 17p

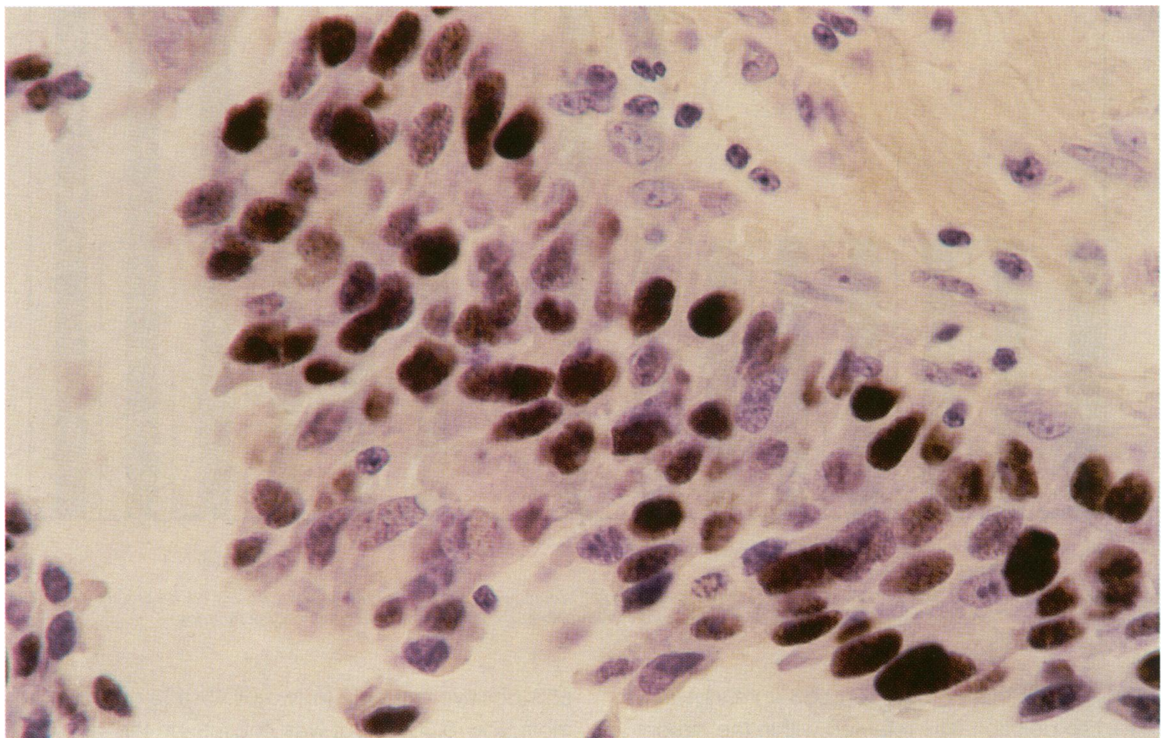

Figure 4. *Immunohistochemical detection of p53. Bladder tumor with p53 positivity in 60 to 80% of nuclei.*

deletions in pTa tumors was also expected, because only five cases with 17p deletion have been reported among 30 tumors in three different studies.[42–44] Habuchi et al[42] recently examined 23 pTa/T1 tumors and did not find a difference in 17p deletion prevalence between pTa (deletion in two of 11 cases) and pT1 tumors (two of 12) and suggested that 17p deletion is associated with development of muscle invasion. In contrast, we found a marked difference in the prevalence of 17p deletion between pTa (four of 43) and pT1 tumors (17 of 42; $P = 0.0004$), and no significant difference between pT1 and pT2-4 tumors (31 of 62). Because 17p deletion was also found by LOH in pT1 bladder tumors in other studies,[43–46] it seems that loss of genetic information on chromosome 17p coincides with development of an invasive phenotype in bladder cancer, including early invasion (pT1).

A strong association was seen between p53 immunostaining and 17p deletions ($P = 0.0005$). However, there was still disagreement between immunohistochemical p53 positivity and physical 17p deletion in 34 of 106 cases. Because immunohistochemically silent p53 mutations have been reported,[47] it is likely that stop or intron mutations are present in some or all of the 10 cases with 17p deletions but no detectable p53 positivity. Also, given the close association of p53 immunostaining and mutation,[8–11] it is likely that there is mutation without 17p loss in many of the 24 cases with positive p53 immunostaining and no deletion. These cases might represent mutations involving both alleles, dominant acting p53 mutations, or tumors with recessive acting p53 mutation in which a loss of the second allele has not yet occurred. Because the number of cases with p53 protein expression but no deletion or LOH on 17p was considerably larger in our study (23%) than in a previous study comparing p53 expression with LOH at 17p (9%) in bladder cancer,[21] methodological differences between RFLP analysis and FISH must also be considered.

There was agreement between FISH and LOH analysis in 11 of 14 cases. The three discrepancies between FISH and LOH analysis illustrate advantages and limitations of both methods. The presence of large numbers of non-neoplastic cells may obscure true allelic loss when total DNA is examined by RFLP analysis. In one case, two centromeres 17 and one p53 signal were seen in 90% of cells by FISH, yet no LOH was detected by RFLP analysis. We conclude that in this case, DNA was extracted from a tissue sample predominantly containing non-neoplastic cells, or else significant heterogeneity between tumor samples led to the discrepancy. On the other hand, RFLP analysis provides data about the quantitative relationship between parental alleles at an examined locus. In one case, a 90% loss of one allele was found by RFLP analysis but no deletion was seen by FISH. This case showed a trisomy 17 in the majority of cells, and it must be concluded that the p53 locus on all three chromosomes was derived from the same parental chromosome. Mitotic recombination of a chromosomal fragment or endoreduplication of an entire chromosome are also possible explanations for a loss of genetic information not detectable by FISH. Because of the inherent admixture of non-neoplastic cells in tumor samples, a decrease of 35 to 40% in signal intensity of one allele is usually considered diagnostic for a deletion in RFLP analysis. However, if there is little admixture of non-neoplastic cells, a chromosomal imbalance such as a trisomy can result in allelic imbalance of up to 50%. This mechanism is possible for the third discrepant case showing trisomy 17 without 17p deletion in FISH examination but LOH of 60% by RFLP analysis. Considering the high frequency of polysomy 17 in high-stage bladder cancer, it is possible that allelic imbalance is responsible for the slightly higher prevalence of 17p deletions in pT2-4 tumors in previous studies using LOH (sum of four studies: LOH in 60 of 92 tumors, 65%)[43–46] as compared to our finding by FISH (29 of 58, 50%).

Another mechanism leading to allelic loss without deletion detected by FISH is loss of an entire chromosome 17 in an aneuploid tumor. Loss of two chromosomes derived from the same parent in a tetraploid tumor will show two centromeres 17 and two p53 signals by FISH, and LOH by RFLP analysis. However, our results suggest that a relative loss of chromosomes 17 is not frequent in bladder cancer, inasmuch as only one case of a monosomy 17 was detected. Also, parallel analysis of chromosome 7 copy number showed only rare instances of fewer 17 copies than seven copies (data not shown). Analysis of ploidy by flow or image cytometry would be useful to define relative chromosome losses in this context.

There is considerable disagreement in the literature about the relationship between LOH at 17p13 and p53 protein expression or mutations.[24–27,45,48–49] In the present study, a correlation between 17p deletion and p53 expression was found in all tumors and in the subgroup of 25 pT1 tumors. However, we did not see a relationship in the subgroup of 40 pT2-4 tumors. This finding might represent the biological variability of p53 alterations. It is possible that recessive p53 mutations, requiring the loss of the second allele for a selective advantage to occur, are more frequent in pT1 tumors, because p53 mutations are frequently associated with p53 deletions in these tumors. Dominant acting p53 mutations that exert their

influence without a complementary 17p deletion might be more common in pT2-4 tumors, as no association between p53 expression and 17p deletions are seen in these tumors. It is also possible that mutations involving both alleles or immunohistochemically silent p53 mutations are more frequent in advanced bladder tumors. The lack of association between p53 protein expression and 17p deletion in pT2-4 tumors might also be due to the presence of another tumor suppressor gene on 17p, as recently proposed in breast and brain tumors.[31–33] However, we found no evidence supporting the existence of a second tumor suppressor gene distal to p53 on 17p in bladder cancer, inasmuch as no deletions were found including C144 on distal 17p that did not also include the p53 locus.

One advantage of FISH analysis over RFLP analysis is that chromosome copy number is characterized simultaneously. Because there was a strong correlation between chromosome 17 copy number and tumor grade and stage, chromosome 17 copy number deserves further evaluation as a prognostic marker. The strong relationship of polysomy with p53 protein expression or with 17p deletion is consistent with the recent hypothesis that the p53 gene, functioning as a "guardian of the genome," might help to protect a cell from genetic damage, including polysomy.[5]

Although there remains considerable debate on this point, pTa and pT1 tumors are frequently grouped into a class of superficial bladder cancer, as opposed to muscle invasive T2-4 tumors.[50,51] Our finding that differences in the presence of 17p deletions and polysomy are much more prominent between pTa and pT1 tumors than between pT1 and pT2-T4 tumors suggests that pT1 tumors are biologically closer to pT2-T4 than to pTa tumors. The high prevalence of 17p alterations and polysomy 17 in pTa/T1 bladder tumors, which have a more unpredictable clinical outcome, makes these parameters promising candidates for prognostic markers. This is especially true because FISH is a simple method requiring very little material with the potential for routine application.

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