

Alterations of the 9p21 and 9q33 Chromosomal Bands in Clinical Bladder Cancer Specimens by Fluorescence *In Situ* Hybridization¹

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ABSTRACT

Purpose: To better define cytogenetic mechanisms of *CDKN2* loss at 9p21 and of *DBCCR1* loss at 9q33 in bladder cancer, and to determine correlation with p53 and pRb.

Experimental Design: Two-color fluorescence *in situ* hybridization (FISH) using a chromosome 9 centromeric probe and locus-specific probes was performed. p53 and pRb were assessed by immunohistochemistry.

Results: Thirty-seven of fifty-five (67%) samples exhibited 9p21 loss, and 32 of 44 (73%) exhibited 9q33 loss. Twelve of 43 informative samples exhibited only 9p21 loss (5 cases) or only 9q33 loss (7 cases). Homozygous deletions were noted at 9p21 and 9q33 in 31 and 14% of cases, respectively, but 9q33 homozygous deletions were generally observed in only a minor clone. There was no correlation of any chromosome 9 loss with stage, but stage did correlate with chromosome 9 ploidy status; aneusomy 9 was observed in 33% of T_a lesions and 71% of more advanced cases ($P = 0.01$). Aneusomy 9 was loosely correlated with p53 abnormalities ($P = 0.07$), but no correlation between any chromosome 9 and pRb abnormalities was discerned.

Conclusions: This study strengthens the proposition that chromosome 9 losses occur early in bladder oncogenesis and before p53 alterations or development of aneusomy. The correlation of aneusomy 9 with p53 abnormalities is consistent with the presumed role of p53 in maintaining cytogenetic stability. Although the observed homozygous deletions

strengthen the hypotheses that *CDKN2* and *DBCCR1* are important tumor suppressor genes, there is no evidence that either is a more critical or an earlier target for oncogenesis.

INTRODUCTION

Cytogenetically determined loss of chromosome 9 was the first and most common genetic loss identified in bladder cancers (1). This observation has since been extended through LOH³ studies, which reveal that LOH for chromosome 9 markers occurs in ~70% of cases (2–5). In the majority of tumors, all informative markers exhibit LOH, suggesting that an entire chromosome 9 has been deleted. Fine mapping of tumors with smaller deletions has helped identify the *CDKN2A* gene on 9p21 as the principal target gene on the short arm (6–11). *CDKN2A* encodes two alternatively spliced proteins, p16 and p14^{ARF} (p19^{ARF} in murine systems). These two proteins are encoded in different reading frames and thus have no homology. They are, however, both important in cell cycle regulation and cellular senescence, with p16 a critical component of the pRb pathway and p14^{ARF} a critical component of the p53 pathway (12–14). A number of mechanisms for *CDKN2A* inactivation in bladder cancer have been described including hemizygous deletion and point mutation of the remaining allele, homozygous deletion, and promoter methylation (6–11).

It is likely that several target genes on the long arm of chromosome 9 are important in bladder cancer oncogenesis, with one potential candidate being *DBCCR1* on 9q33 (15). Although the function of this gene is not clear, it is in a common region of hemizygous deletions, and both homozygous deletions and methylation-based silencing have been described (15, 16).

Despite extensive work with LOH studies and identification of putative tumor suppressor genes, a number of relevant questions regarding chromosome 9 deletions in clinical bladder cancer remain: (a) it remains unclear whether deletions on the long or short arm occur in any particular sequence; (b) the incidence of homozygous deletions, especially at 9q33, is not well known. This occurs because contamination with a relatively small population of normal cells or tumor heterogeneity in which only a small clone of cells has a homozygous deletion obscures detection of such deletions with typical DNA-based studies; and (c) LOH studies will reveal loss of an allele but will give no information as to the ploidy status of the chromosomes. Ploidy status remains important because it may provide information on the mechanisms for loss, may provide additional prognostic information for the tumor natural history, and perhaps most importantly may allow interpretation of LOH studies

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³ The abbreviations used are: LOH, loss of heterozygosity; FISH, fluorescence *in situ* hybridization; PAC, PI artificial chromosome.

that reveal an allelic imbalance rather than outright loss of an allele.

Some of these issues can be addressed by FISH studies. A number of such studies have already been published (17–23). The majority have used only a centromeric probe specific for chromosome 9. Those studies that have used specific probes to genetic regions have not examined the putative 9p21 and 9p33 tumor suppressor genes simultaneously, and thus the relationship of alterations in these regions is unknown. Finally, FISH-determined aberrations have generally not been analyzed in concert with evaluation of other known genetic alterations, of which p53 and pRb are likely the most important to bladder oncogenesis. This is especially critical because pRb alterations generally occur only in tumors without p16 alterations (and *vice versa*). In addition, aneuploidy in general, which may or may not be specifically related to chromosome 9 aneuploidy, is correlated with p53 alterations in many cancers, including bladder cancer (24, 25).

To address these issues, we analyzed FISH-determined chromosome 9 alterations in a series of bladder cancers by using touch preparations of fresh as well as frozen clinical specimens and correlated our findings with pRb and p53 alterations, as determined by immunohistochemistry. The use of touch preparations was chosen to circumvent difficulties in interpretation attributable to sectioned nuclei and lower hybridization efficiencies in standard pathological specimens. We performed two-color FISH with a chromosome 9 centromere probe and a PAC specific for the *CDKN2* region, as well as a chromosome 9 centromere probe and a PAC specific for the *DBCCR1* region. We confirm that chromosome 9 loss is present in the majority of samples and that there is no correlation with stage. We also show that both 9p21 and 9q33 homozygous deletions are detectable, lending further support to the hypothesis that 9q33 contains an important tumor suppressor gene. Finally, we demonstrate that chromosome 9 aneuploidy is common and correlates with p53 abnormalities.

PATIENTS AND METHODS

Patients and Samples. Investigations reported here were approved by the University of Chicago Institutional Review Board. All tumor samples were obtained from patients undergoing clinically indicated cystoscopic resection or cystectomy for bladder cancer. For fresh samples, tissue in normal saline was immediately transported to the research laboratory after the resection. Touch preparations were made by gently pressing the urothelial surface of the specimen on standard microscope slides. The sample was then immediately transported to the pathology laboratory for standard pathological analysis. Slides were allowed to dry for 3–5 min and then fixed and processed for FISH analysis. Frozen samples were generally collected from obvious tumor regions from cystectomy specimens in the pathology laboratory, embedded in tissue freezing medium (Triangle Biological Sciences, Durham, NC), flash frozen in liquid nitrogen, and stored at -80°C for up to 5 years prior to use. To process for FISH, a portion of the sample was thawed in PBS at room temperature, and touch preparations were prepared as above. For normal control urothelial samples, we used anonymous sections of ureter from patients undergoing a nephrectomy

who had no evidence of transitional cell cancer. These anonymous samples were all removed for clinically indicated reasons and destined to be discarded. Touch preparations of the urothelial surface was performed in the same manner as above. A total of 55 tumor samples were examined with the following stages: 18 T_a (1 ungraded, 10 grade 1, and 7 grade 2), 7 T_{cis} , 10 T_1 (1 grade 1, 4 grade 2, and 5 grade 3), and 21 invasive cancers, of which 2 had positive nodes. Because of limited sample in some cases, not all specimens were used for both 9p21 and 9q33 analysis (see “FISH” and “Results” below).

FISH. Slides with dried touch preparations were washed in PBS at 37°C for 10 min, and cells were lysed with 0.075 M KCl for 10 min at 37°C . After a brief rinse in distilled water, slides were fixed for 10 min in fresh 3:1 methanol:glacial acetic acid at room temperature. If not immediately used for hybridization, slides were dried and stored at -20°C for up to 12 months. Immediately prior to hybridization, slides were treated with 130 $\mu\text{g/ml}$ RNase in $2\times$ SSC at 40°C for 1 h; washed sequentially in $2\times$ SSC and 70, 80, and 95% ethanol at room temperature; and then denatured at 70°C in 70% formamide/30% $4\times$ SSC for 2 min prior to washing in graded alcohols a second time. Slides were kept at 40°C until hybridization was completed.

The used probes were a commercial directly labeled (SpectrumOrange) 9 centromere probe (CEP9; Vysis, Downers Grove, IL), a 9p21 PAC (76I15) isolated from a commercial human PAC library (Incyte Genomics, Palo Alto, CA), and a 9q33 PAC (280C3) spanning the *DBCCR1* locus obtained from M. Knowles (Imperial Cancer Research Fund Cancer Medicine Research Unit, St. James' University Hospital, Leeds, United Kingdom Britain). Locus specificity was confirmed on a regular basis throughout the period of study by FISH hybridization to metaphase spreads of normal human lymphocytes. PACs were labeled by nick translation with inclusion of biotin-labeled dUTP (Boehringer Mannheim). Selected samples underwent three-color FISH, in which 9p21 and 9q33 probes were nick translated separately with biotin- and digoxigenin-labeled dUTP, respectively, and then used with a directly labeled SpectrumAqua 9 centromere probe (CEP9 9SA; Vysis).

Probes were detected by incubation with avidin-FITC or anti-digoxigenin-rhodamine, and counterstaining was performed with 4,6-diamidino-2-phenylindole. Image capture for presentation purposes was performed with a Photometrics CCD camera, and image enhancement was performed with Adobe Photoshop version 4.0. Fifty to 200 cells with intact nuclei, as assessed by 4,6-diamidino-2-phenylindole staining, from each sample were counted. Twenty-two % of the samples were assessed independently by two observers, and the results were averaged. Interobserver variability was low, and disagreement over classification of a tumor sample as “abnormal” in any parameter, as defined in “Results” below, occurred in only 2 cases. In both cases, one observer noted homozygous 9p21 deletion in a minor clone, whereas the other observer noted only hemizygous losses.

Immunohistochemistry. Slides were prepared from paraffin-embedded blocks confirmed to contain tumor on standard H&E staining. Serial sections were subjected to antigen retrieval in citric acid buffer (pH 6.0) and hybridized with the primary antibody at 4°C overnight. For p53 staining, 1 $\mu\text{g/ml}$ antibody

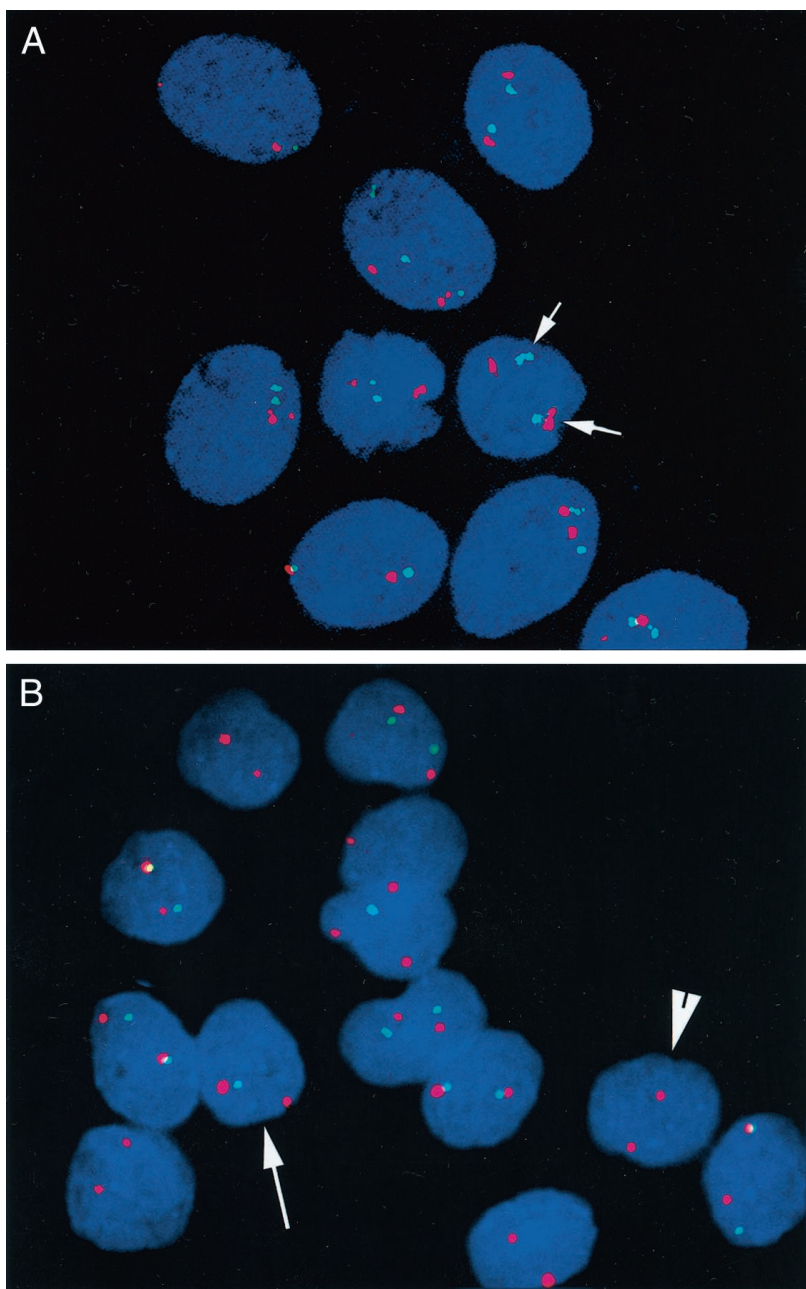


Fig. 1 Representative examples of FISH staining for 9 centromere (red) and 9p21 (green) in a normal ureter (A) and a tumor (B). Doublet signals (arrows in A), likely representing hybridization to two chromatids from a single chromosome, where counted as one signal. In B, the arrowhead shows a cell with evidence of 9p21 homozygous deletion, and the arrow shows a cell with a 9p21 hemizygous deletion.

2 (clone 1801; Oncogene Research, Boston, MA) or 0.5 $\mu\text{g/ml}$ antibody 6 (clone DO1; Oncogene Research Products, Boston, MA) were used. For pRb staining, 0.5 $\mu\text{g/ml}$ G3-245 (PharMingen, San Diego, CA) was used. Visualization was accomplished by hybridization with biotinylated antimouse IgG (Vector Laboratories), followed by incubation with avidin-labeled horseradish peroxidase and using 3,3'-diaminobenzidine as a chromagen (Vector Laboratories). All slides were counterstained with hematoxylin.

Tumors were considered positive for p53 (interpreted as mutant p53) if $>10\%$ of the tumor cells stained and were considered negative (interpreted as wild-type p53) if $<10\%$ of

the tumor cells stained. Differences in staining pattern between the two p53 antibodies generally consisted of weak staining with the DO1 antibody and absent staining with the 1801 antibody. These cases were all considered to be negative (interpreted as wild-type p53). As reported previously, slides stained for pRb were scored as absent staining (0), normal staining (1+), or intense staining (2+; Ref. 26).

RESULTS

Chromosome 9 FISH: Normal Controls. To characterize our FISH assay, we determined hybridization patterns in 25

Table 1 FISH results for normal control ureters

Homo. 9p21 and homo. 9q33 indicate the percentage of cells with detectable centromeric signal but no specific regional signal. 9p21 < 9c and 9q33 < 9c indicate the percentage of cells with fewer specific regional signals than centromeric signals. 9c = 1 and 9c > 2 indicate the percentage of cells with monosomy 9 or aneusomy 9 as detected by the centromeric signal.

| | Homo. 9p21 | 9p21 < 9c | Homo. 9q33 | 9q33 < 9c | 9c = 1 | 9c > 2 |
|--------|---------------|-----------|---------------|--------------|--------|--------|
| Median | 0.0 | 1.0 | 0.0 | 1.5 | 1.0 | 1.0 |
| Mean | 0.2 | 1.4 | 0.8 | 1.9 | 0.8 | 1.3 |
| SD | 0.4 | 1.5 | 2.3 | 1.9 | 0.8 | 1.7 |

independent, anonymous, normal ureter samples. Normal samples were randomly interspersed with the tumor samples. Of these, 22 were analyzed with combined CEP9/9p21 probes, 15 were analyzed with combined CEP9/9q33 probes, and 12 were analyzed with both sets of probes. Fig. 1A shows an example of a CEP9/9p21 FISH of a normal sample. Chromosome 9 deletion characteristics were categorized as 9p21 homozygous deletions (no 9p21 signal in the presence of at least one chromosome 9 centromere signal), 9p21 signals less than 9 centromere signals, 9q33 homozygous deletions, and 9q33 signals less than 9 centromere signals. Table 1 shows a summary of the results and indicates that hybridization and detection efficiency exceeded 95%.

Chromosome 9 FISH: Tumor Samples. On the basis of the results from FISH on normal samples, we defined an abnormal tumor sample as one in which the fraction of cells with any deletion was >7%. This corresponds to 3 SDs above the mean in control samples for the most variable chromosome 9 characteristics, *i.e.*, the fraction of homozygous 9q33 deletions. Fig. 1B shows an example of a tumor sample with 9p21 deletions, and Fig. 2 shows the proportion of samples with each of the delineated chromosome 9 abnormalities noted. One result of our counting mechanism is that some of the abnormal samples had only a minor population of cells exhibiting the specific chromosome 9 abnormality. However, because no effort was made to enrich the samples for tumor cells beyond simple gross inspection, we felt that this counting mechanism was a more sensitive manner in which to detect these changes. Similar to previous studies using both LOH and chromosomal techniques, 37 of 55 (67%) of samples exhibited relative loss of 9p21 signals over chromosome 9 centromere signals, and 32 of 44 (73%) of samples exhibited relative loss of 9q33 signals. There was no statistical difference in incidence of these findings between stages.

Of 43 samples for which there were data on both 9p21 and 9q33 hybridizations, 31 (72%) had concordant findings (*i.e.*, underrepresentation in reference to the chromosome 9 centromere probe at both loci or neither locus). In five samples, there was evidence of underrepresentation of the 9p21 but not the 9q33 locus, and in 7 samples, there was evidence of underrepresentation of the 9q33 but not the 9p21 locus.

Homozygous deletions were also detected at both the 9p21 as well as the 9q33 loci at a frequency of 31% (17 of 55) and 14% (6 of 44) with no statistical difference between the stages. Notably, however, 9q33 homozygous deletions always occurred

in a minor clone of cells (typically 10–20% of the sample), whereas 9p21 homozygous deletions could be found in up to 78% of the cells in a sample. Monosomy 9 (as defined by only 1 centromeric signal) was found in only a minority of samples with a higher incidence in T_a than all other stages (22% *versus* 5%, two-sided *P* = 0.08). Conversely, aneusomy 9 (as defined by greater than two centromeric signals) occurred less frequently in T_a than in all other stages (33% *versus* 71%, two-sided *P* = 0.01). It is important to note that although 72% of all samples had evidence of some 9p loss (monosomy 9 or underrepresentation of 9p21 relative to CEP9 or 9p21 homozygous deletion), only 3 of 55 samples exhibited monosomy 9 as the sole evidence of this loss.

Immunohistochemistry for p53 and pRb. Table 2 gives results of p53 and pRb staining by stage. Absent pRb staining was noted in 1 of 14 T_a samples *versus* 9 of 29 samples with higher stages (*P* = 0.13, two-sided Fisher's exact test). If overexpression of pRb is also classified as abnormal (26), 5 of 14 T_a and 15 of 29 higher stage samples were considered to be altered (*P* = 0.35, two-sided Fisher's exact test). As in previous studies, p53 positivity correlated with stage (*P* = 0.06, one-sided Fisher's exact test for pT_a *versus* all others). Because p53 abnormalities have been correlated with aneuploidy (24, 25) we examined the correlation of p53 immunohistochemistry with chromosome 9-specific aneuploidy (Table 3A). There is a borderline significant trend of increasing aneuploidy with p53 positivity (*P* = 0.07, two-sided Fisher's exact test). Finally, because p16 abnormalities have been correlated with increased expression of pRb and *vice versa* (27, 28), we examined the correlation of pRb expression with any 9p21 abnormality (9p21 homozygous deletion, 9p21 underrepresentation, and monosomy 9). Table 3B reveals that there is no obvious relationship between these findings in aggregate (or individually) with pRb immunohistochemistry.

DISCUSSION

As in previous studies, we confirm that chromosome 9 abnormalities are a common abnormality in bladder cancer and that there is no relationship between chromosome 9 loss and stage (Fig. 2), suggesting that this loss is an early event in bladder oncogenesis (2, 5, 8, 9, 11, 17, 19, 23). It is important to note that chromosomal losses as assessed in this and other FISH studies are not necessarily equivalent to losses as assessed by LOH studies. The latter method assesses relative levels of paternal and maternal alleles within a tumor but is unable to determine chromosomal number or structure. FISH studies, on the other hand, although able to assess aneusomy and are more sensitive for detecting subtle underrepresentation of a region (*e.g.*, three specific region signals compared with four centromere signals), are unable to detect relative allelic contributions to the visualized chromosomes. Thus, allelic loss followed by chromosomal duplication may not be detectable with such methods. Despite these differences, 60–70% of bladder cancer samples can be demonstrated to have chromosome 9 losses whether such losses are assessed molecularly or chromosomally.

The 20–40% incidence of 9p21 homozygous deletions observed here is similar to that observed in other FISH studies with bladder cancer specimens (23) but higher than that ob-

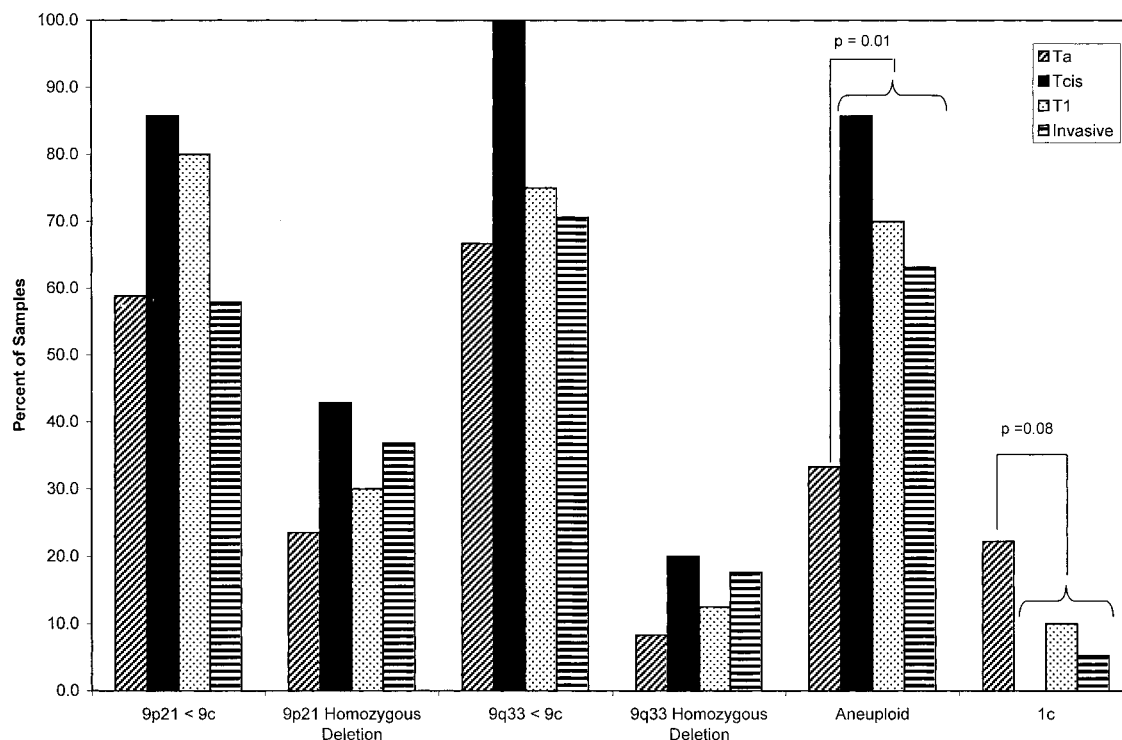


Fig. 2 Chromosome 9 abnormalities in tumor samples by stage. 9p21 < 9c and 9q33 < 9c, percentage of cells with fewer specific regional signals than centromeric signals; 9p21 Homozygous Deletion and 9q33 Homozygous Deletion, percentage of cells with detectable centromeric signal but no specific regional signal; Aneuploid, more than two centromeric signals; 1c, only one centromeric signal.

Table 2 p53 and pRb immunohistochemistry staining results by stage

Fisher's exact test for association of p53 with stage reveals $P = 0.06$. An association between stage and pRb staining is not evident.

| | p53 (n = 43) | | pRB (n = 43) | | |
|------------------|--------------|----------|--------------|--------|-----------|
| | Negative | Positive | Normal | Absent | Increased |
| T _a | 10 | 4 | 9 | 1 | 4 |
| T _{cis} | 4 | 3 | 5 | 1 | 1 |
| T ₁ | 3 | 5 | 4 | 2 | 2 |
| Invasive | 4 | 10 | 5 | 6 | 3 |

served with molecular studies (11, 29). This difference is likely attributable to technical issues including the ability of FISH to detect smaller subpopulations. Homozygous deletion of the *DB-CRR1* locus has been reported, but only infrequently (16), and was observed in only 10–20% of the samples in this study. The fact that it was observed in only a minor clone, even when detected, raises the issue of whether this observation was artifactual. However, a sample was classified as harboring a 9q33 homozygous deletion only if the fraction of cells with such a deletion was 3 SDs greater than the fraction observed in control transitional epithelial samples. Although minor differences between characteristics of the control and tumor samples could potentially account for the losses, this is thought to be unlikely. It is interesting to speculate on the reasons that only a minor clone harbors 9q33 deletions, but we suspect that a homozygous deletion inactivates not only the target tumor suppressor gene

Table 3 p53 and pRb immunohistochemistry staining by chromosome 9 aneuploidy and 9p21 abnormalities, respectively

In A, $9c \leq 2$ refers to samples with no evidence of chromosome 9 aneuploidy, whereas $9c > 2$ refers to samples with evidence of chromosome 9 aneuploidy. Fisher's exact test for association of p53 staining with chromosome 9 ploidy reveal $P = 0.07$. In B, 9p21 normal refers to samples in which the number of 9p21 signals was equal to the number of chromosome 9 centromere signals, and 9p21 abnormal refers to samples in which there was a relative loss or evidence of monosomy 9. There is no statistically significant relationship between pRb immunohistochemistry and any chromosome 9 loss.

A. p53 vs. chromosome 9 ploidy

| | p53 normal | p53 increased |
|-------------|------------|---------------|
| $9c \leq 2$ | 13 | 7 |
| $9c > 2$ | 8 | 15 |

B. pRb vs. chromosome 9 deletions

| | 9p21 normal | 9p21 abnormal |
|---------------|-------------|---------------|
| pRb nl | 7 | 16 |
| pRb absent | 2 | 8 |
| pRb increased | 3 | 7 |

but also other genes or regulatory regions that fail to provide a growth advantage to cells containing such deletions. Nonetheless, 9q33 homozygous deletions lend further credence to the hypothesis that a gene localized to this region, such as the proposed *DBCCR1* gene, is a bona fide tumor suppressor gene.

Our studies also confirm that the majority of cases, even

the early T_a lesions, have evidence of losses on both arms of chromosome 9. We were able to detect samples as well as individual cells within samples that had evidence of only 9p21 or 9q33 underrepresentation but were unable to detect any predominance of one over the other. There is thus no evidence that either of the two loci is an “earlier” event in bladder oncogenesis. Examination of premalignant lesions may be required to distinguish the order of 9p and 9q deletions.

The observation of chromosome 9 aneuploidy in 33% of T_a lesions and 71% of all other lesions (Fig. 2) is similar to flow studies (30, 31) as well as other chromosome 9 FISH studies (32). It is important to note, however, that aneuploidy of one chromosome does not necessarily reflect aneuploidy in general (33). More general aneuploidy and chromosomal instability have been associated with p53 abnormalities, and our studies suggest that such a correlation may extend to specific chromosome 9 aneuploidy as well. We also note that monosomy 9 is clearly more common in the early T_a tumors, whereas aneusomy 9 is more common in T_{cis} and more advanced lesions (Table 3). This lends further molecular support to the clinical observation that T_a tumors as a group are distinct from the other noted bladder cancers.

Although *in vitro* studies have suggested that 9p21 and resultant p16 abnormalities correlate inversely with pRb status, we were unable to detect such a correlation in a limited subset of our samples (Table 3). Whether this is attributable to insensitivity of the FISH technique for detecting all types of p16 alterations, insensitivity of our immunohistochemical analysis for pRb overexpression or some combination of factors remains unclear.

In sum, we confirm previous observations that chromosome 9 alterations are an early and frequent abnormality in bladder cancer and that both 9p21 and 9q33 losses are important. The lack of predominance in deletions on one arm over the other and the rarity of samples with deletions on only one arm, even in the earliest stage tumors, strongly suggest that extensive chromosome 9 losses are necessary for the full oncogenic phenotype in bladder cancer and that neither of the two presumed target genes is a more critical “gatekeeper.” Nonetheless, the observation of 9q33 homozygous deletions further strengthens the hypothesis that *DBCCR1*, or another gene in this region, is an important tumor suppressor gene for this malignancy. Finally, we show that chromosome 9 aneusomy is frequent and that p53 overexpression by immunohistochemistry correlates with such findings. Given the correlation of these findings with tumor stage strengthens the molecular progression model in bladder cancer in which inactivation and loss of genes on chromosome 9 precede development of p53 abnormalities (34). This small and heterogeneous patient cohort did not allow us to correlate outcome with any of the observed molecular findings. The clinical prognostic and predictive implications of FISH-determined chromosome 9 alterations thus remain the subject of current investigations.

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