Identification of Chromosome 9 Alterations and p53 Accumulation in Isolated Carcinoma in Situ of the Urinary Bladder versus Carcinoma in Situ Associated with Carcinoma

Anton H. N. Hopman,* Miriam A. F. Kamps,* Ernst J. M. Speel,* Rene F. M. Schapers,† Guido Sauter,‡ and Frans C. S. Ramaekers*

From the Department of Molecular Cell Biology,* Research Institute of Growth and Development, University of Maastricht, Maastricht, The Netherlands; the Department of Pathology,† Sichting Ziekenhuizen Noord Limburg, Venlo, The Netherlands; and the Institute of Pathology,‡ University of Basel, Basel, Switzerland

Carcinoma in situ (CIS) of the urinary bladder is a flat, aggressive lesion and may be the most common precursor of invasive bladder cancer. Although chromosome 9 alterations are among the earliest and most prevalent genetic alterations in bladder cancer, discrepancy exists about the frequency of chromosome 9 losses in CIS. We analyzed 22 patients with CIS of the bladder (15 patients with isolated CIS, 7 patients combined with synchronous pTa or pT1 carcinomas) for gains and losses of chromosome (peri)centromere loci 1q12, 7p11-q11, 9p11-q12, and 9p21 harboring the INK4A/ARF locus (p16INK4A/p14ARF) and INK4B (p15INK4B) by multiple-target fluorescence in situ hybridization, and for p53 protein accumulation by immunohistochemistry. In 15 of 20 (75%) CIS lesions analyzed p53 overexpression was detected, whereas aneusomy for chromosomes 1 and 7 was identified in 20 of 22 (91%) CIS. In 13 of 22 (60%) CIS cases analyzed, 12 of which were not associated with a synchronous pTa or pT1 carcinoma, no numerical losses for chromosome 9 (p11-q12 and 9p21) were detected as compared with chromosomes 1 and 7. Furthermore 9 of 12 (75%) patients showed a metachronous invasive carcinoma within 2 years. In the remaining nine biopsies CIS lesions (40%) were recognized that showed losses of chromosome 9p11-q12 and 9p21, six of these were associated with a synchronous pTa or pT1 carcinoma. Three of these carcinomas were pTa and exhibited loss of 9q12 as well as a homozygous deletion of 9p21. The others were invasive carcinomas in which CIS lesions were also recognized that showed no numerical loss of chromosome 9, but did show an accumulation of p53. In conclusion our data demonstrate that predominantly isolated CIS lesions contained cells with no specific loss of chromosome 9, as opposed to CIS lesions with synchronous carcinomas that showed evidence of chromosome 9 loss. Furthermore our data strengthen the proposition that p53 mutations (p53 overexpression) precede loss of chromosomes 9 and 9p21 in CIS as precursor for invasive bladder cancer, as opposed to noninvasive carcinomas where chromosome 9 (9p11-q12) losses are early and frequently combined with homozygous deletions of 9p21. (Am J Pathol 2002, 161:1119–1125)
have an aneuploid DNA content.\textsuperscript{5,13,14} Controversy exists on the involvement of chromosome 9 in CIS. Rosin and colleagues\textsuperscript{15} found loss of heterozygosity for 9p and q in the majority of CIS cases, including a high frequency of homozygous deletions of the p16 locus. This is opposite to the results of Spruck and colleagues\textsuperscript{16} who identified loss of heterozygosity of chromosome 9 loci in only 12\% of CIS and dysplastic lesions. One possible explanation for these inconsistent data would be a selection of different types of CIS in previous studies. For example, it would be possible that genetic differences exist between isolated CIS and CIS occurring adjacent to carcinomas. To address these questions we used fluorescence in situ hybridization (FISH) for in situ detection of genetic alterations in the histological context, with the advantage that information about the ploidy of chromosomes can be obtained, grade transitions can be analyzed in the biopsy, and small areas of the lesion can be informative.\textsuperscript{9}–\textsuperscript{11} Our comparison of solitary CIS with CIS adjacent to pTa and pT1 carcinomas provided evidence for genetic differences in chromosome 9 alterations in CIS with and without adjacent carcinomas.

Materials and Methods

Tissue Material

Formalin-fixed, paraffin-embedded biopsy specimens from 22 patients were selected from a series of \textasciitilde900 bladder tumor samples collected in the past 10 years. Selection was based on the availability of a CIS fragment in the tissue blocks. The patients showed no past history of invasive carcinoma or CIS in 14 cases, CIS in 6 cases, and invasive carcinoma in 2 cases. At the time of diagnosis the tissue block showed no synchronous pTa or pT1 carcinoma in 15 cases and concomitant carcinoma in 7 cases. Two pathologists (RS, GS) reviewed the slides. Staging and grading was performed according to the World Health Organization.\textsuperscript{3} The patient history and histopathological classifications are presented in Figure 1.

Figure 1. Histopathology, clinical data, and p53 immunohistochemical genetic analyses of CIS from 22 patients. a: no p53 accumulation; b: p53 accumulation; c: heterogeneity with respect to accumulation (no diffuse pattern, but distinct separate histological areas). d: disomy for 1c and/or 7c; e: aneusomy for chromosomes 1 and/or 7; f: balanced chromosome copy numbers for 9c and 9p21 as compared with 1c and/or 7c copy numbers; g: loss of 9c and/or 9p21 (lower copy numbers). Copy numbers are estimated on basis of number of FISH signals. h: Heterogeneity with respect to loss. i: HZD, homozygous deletion of 9p21; j: no HZD. k: HZD. l: Extreme large nuclei were recognized indicating polyploidization of aneuploid cells. m: Trisomy for 9c and 9p21. n.e.: not evaluated. cyst.: cystectomy.

\textsuperscript{p53 Immunohistochemistry}

Four-\mu m-thick tissue sections were deparaffinized and pretreated with 0.3\% H\textsubscript{2}O\textsubscript{2} in methanol to quench endogenous peroxidase activity, followed by antigen retrieval using microwave heating in 0.01 mol/L of citrate buffer (pH 6.0) for 30 minutes.\textsuperscript{17} Both normal and mutant p53 protein were detected by incubating the slides subsequently with a monoclonal anti-p53 antibody (1:50, clone DO-7; DAKO A/S, Glostrup, Denmark), biotinylated rabbit anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA) and an avidin-biotinylated peroxidase complex ( Vectastain PK4000, Vector Laboratories). Peroxidase activity was visualized using diaminobenzidine/H\textsubscript{2}O\textsubscript{2} and sections were counterstained with hematoxylin and mounted in Entellan (Merck, Darmstadt, Germany). p53 staining was scored visually as negative (no staining) or positive
(clear nuclear staining and negligible background in >30% of the nuclei). Positivity appeared to be strictly limited to atypical cells in cases of CIS.

**FISH**

**Probe Selection and Labeling Procedures**

The probes for chromosome 1 (1q12 indicated as 1c) and 7 (7p11.1-q11.1 indicated as 7c) were selected to identify overall DNA ploidy and chromosome aneusomy. The probes for chromosome 9 (9q12, 9p11-q11 designated as 9c, and 9p21) were chosen to detect monosomy and losses of the inkb4a locus, respectively. The following probe sets were used for multiple target analyses: 1c-Bio, 9c-Dig for 1q12 (pUC 1.77), and 9q12 (pHUR 98). The probes were labeled by standard nick translation with biotin- and digoxigenin-dUTPs. CEP 7 (7p11.1-q11.1; 7c), CEP 9 (9p11-q11), and LSI unique sequence DNA probe for 9p21 were directly labeled with SpectrumGreen, SpectrumRed, and SpectrumGold, respectively (courtesy of Vysis, Downers Grove, IL).

**Tissue Pretreatment**

FISH was performed on 4-μm-thick tissue sections. FISH was performed as described previously. Briefly, sections were deparaffinized, pretreated with 85% formic acid/0.3% H2O2 for 20 minutes at room temperature, and subsequently dehydrated with 70% ethanol containing 0.1 mol/L imidazole, pH 7.6, and 0.001% H2O2 under a coverslip for 10 minutes at 37°C. Thereafter, the slides were soaked in 5 mmol/L of MgCl2/PBS containing 0.3% H2O2 for 10 minutes at room temperature to block the remaining peroxidase activity. The digoxigenin-labeled probe was detected with peroxidase-conjugated sheep anti-digoxigenin Fab fragments (SHaDIG-PO, 1:100; Boehringer Mannheim, Mannheim, Germany), followed by a tyramide signal amplification using fluorescein-labeled tyramide (see above). Finally, the slides were washed in PBS containing 0.05% Tween-20 (Janssen Chimica, Beerse, Belgium), dehydrated in an ascending ethanol series and mounted in Vectashield (Vector Laboratories, Inc.) containing 4',6-diamino-2-phenyl indole (DAPI) (0.5 ng/μl, Sigma). The tissue sections hybridized with the directly labeled probes were dehydrated after the stringent washing steps and embedded as described above. Microscope images were recorded with the Metasystems Image Pro System (black and white charge-couple device camera; Metasystems, Sandhausen, Germany) mounted on top of a Leica DM-RE fluorescence microscope (Leica, Wetzler, Germany) equipped with fluorescein isothiocyanate, tetramethyl-rhodamine isothiocyanate, DAPI, and SpectrumGold single bandpass filters for single-color analysis and a triple bandpass filter set (fluorescein isothiocyanate, tetramethyl-rhodamine isothiocyanate, DAPI) for simultaneous dual- and triple-color analysis.

**Evaluation of FISH Signals**

Hybridizations on metaphase spreads were used as controls to guarantee probe specificity and hybridizations on tissue sections with proven aneusomies (monosomy, disomy, trisomy, and tetrasomy) to assure interpretation accuracy. Hybridizations on lymphocytes, stromal cells, or endothelial cells were used as internal control to exclude hybridization artifacts and interpretation problems. Evaluation was performed by two investigators (AH and MK) according to the following three subsequent assessments and criteria.

First, in all cases FISH signals were scored per color and nucleus for the presence of aberrant copy numbers. The highest copy number per nucleus was determined and was set when >20% of the nuclei showed this number of FISH signals. Based on this evaluation, histopathologically classified areas (normal, hyperplastic, dysplastic, CIS, pTa, and pT1) were categorized as either monosomic, disomic, trisomic, tetrasomic, or polysomic (more than four signals per cell) for the respective probe targets.

Second, in a subset of cases an estimation of chromosomal losses and gains was determined, taking into consideration the strict criteria for the classification of individual nuclei, by counting the ratio between the number of different chromosomal targets in 50 to 200 nuclei. Balanced chromosome copy numbers will result in a ratio of ~1.0, whereas a monosomy for example will give a ratio of 0.5, and a trisomy a ratio of 1.5. Both analyses confirmed in all cases the scoring and classification data.
Third, loss of chromosomes 9 (regions) was assumed if both analyses showed fewer FISH signals for 9c and/or 9p21 than 1c and/or 7c signals. A homozygous deletion for 9p21 was considered when in epithelial layers no 9p21 signals were seen, while within the same microscopic image, two signals could be detected in lymphocytes, stromal cells, or endothelial cells.

**Results**

**Accumulation of p53 in CIS Lesions**

Cells with a strong nuclear p53 staining reaction were observed in 15 of 22 CIS cases, 2 of which could not be evaluated. Therefore 75% of the analyzed CIS lesions showed p53 overexpression (Figure 1). In these cases the p53 positivity was strictly limited to atypical cells distributed throughout the epithelial cell layers. In two patients (cases 7 and 21) CIS areas were recognized showing heterogeneous p53 staining. The positivity was restricted to areas that contained a chromosomal aneusomy for 1c and 7c (see also below). Areas classified as normal urothelium showed no accumulation of p53 protein. All invasive carcinomas adjacent to CIS showed p53-positive staining patterns.

**Genetic Alterations in CIS Lesions**

**Chromosomes 1 and 7**

The FISH results of CIS lesions are presented in Figure 1 along with the patient data and presence of a synchronous pTa or pT1 carcinoma. In 20 of 22 CIS lesions more than two copies of 1c and 7c per nucleus were observed in the main population of cells, indicating chromosomal aneusomy. In most cases tetrasomy was observed. In seven CIS lesions additional groups of cells were observed that exhibited aberrant copy numbers in the range of 4 to 8, implicating polyploidization of tetrasomic/aneusomic cells.

**Chromosome 9 Alterations**

Two groups of CIS lesions can be recognized with respect to chromosome 9 alterations (Figure 1), ie, those with copy numbers equal to 1c and/or 7c (cases 1 to 13) and those with relative losses in comparison to 1c and 7c (cases 14 to 22).

In the first group, ie, 13 cases showing no loss of chromosome 9, tetrasomies for 9c and 9p21, and sometimes a profound heterogeneity were seen, always following the 1c and 7c copy numbers. Typical examples of FISH on normal epithelium and a CIS lesion are depicted in Figure 2: A to D (case 4). Areas 1 (nest of Brunn) and 2 show normal epithelium containing cell nuclei with two copies for 7c, 9c, and 9p21. The CIS lesion (area 3) showed for all loci a maximum of four copies per nucleus and was classified as tetrasomic. In 12 of these 13 cases no synchronous pT1 carcinoma was detected.

The second group showed CIS lesions with a loss of chromosome 9, as compared to 1c and 7c copy number. Four cases (cases 14 to 17) contained different CIS areas with and without relative numerical loss of chromosome 9, retaining at least one copy of chromosome 9 and 9p21. Representative examples are shown in Figure 2, E to H (case 16), depicting two different p53-positive areas (Figure 2, E and G) exhibiting no loss (Figure 2F) and loss (Figure 2H) of 9c signals. Figure 2F, furthermore shows large nuclei with high copy numbers indicating polyploidization of tumor cells. In both areas the copy numbers for 1c and 9c were equal indicating a tetrasomy for both chromosomes. In three of these four patients a synchronous invasive carcinoma was diagnosed. Five patients (cases 18 to 22) presented with CIS lesions showing in the entire area only loss of chromosome 9. In three of these five cases a synchronous pTa carcinoma was detected. Representative examples of these lesions are shown in Figure 2, I to N, and Figure 3. Figure 2, I to K (case 19), illustrates a lesion with a homoygous deletion of 9p21 in detached cells. This case proved to be p53-negative (not shown). In the basal cell compartment cells with two signals for 7c, 9p21, and 9p21 were recognized. In this small biopsy the urothelium was primarily denuded. In the biopsy of patient 21 papillary noninvasive carcinoma regions with both low and high grading (G2 and G3) were recognized (Figure 2; L to N). Figure 3 provides a schematic overview of the histopathological diversity (Figure 3A) and frequency distributions of the number of FISH signals in the pTaG2, pTaG3, and CIS areas in Figure 3, B, C, and D, respectively. Only in a small region of this case an equivocal CIS was diagnosed. In the coexisting pTaG2 neoplasia disomy for chromosomes 1 and 7 was seen in combination with a monosomy for chromosome 9 (Figures 2M and 3B, homozgosous deletion 9p21 is not shown). The transition pTaG2 to pTaG3 was characterized by a duplication of the copy number for chromosomes 1, 7, and 9 (Figures 2N and 3C). On development to CIS the genetic pattern becomes more complex in which chromosome 9 is still underrepresented.

Our results demonstrate that a significant correlation exists between losses of chromosome 9 in CIS lesions and the presence of a synchronous carcinoma, ie, in 6 cases with loss of chromosome 9 a synchronous carcinoma was diagnosed, whereas in 13 cases without loss only in 1 case a concomitant carcinoma was detected (Fisher’s exact test, $P < 0.01$; Figure 1). Nevertheless, it should be realized that in most solitary CIS lesions clear signs of genetic instability were observed, such as p53 accumulation, chromosome aneusomy, and polyploidization. This might explain that six patients with a solitary CIS were diagnosed for a metachronous carcinoma within 2 years.

**Discussion**

Loss of chromosome 9 (regions) is one of the most frequent genomic alterations known in bladder cancer, and...
has been reported to be detectable in early premalignant lesions such as hyperplasia and CIS, considered to be precursors of papillary and invasive carcinomas, respectively. However data with respect to chromosome 9 in CIS are scarce and contradictory. In this study chromosome 9 alterations were investigated by a highly sensitive FISH approach comparing copy numbers for several chromosome 9 loci with chromosome 1 and 7 copy numbers. Two groups of CIS lesions were recognized, ie, those with chromosome 9

**Figure 2.** FISH for chromosomes 7, 9, and 9p21, and p53 immunohistochemistry of tissue sections from patients diagnosed with CIS of the bladder. A–D: Case 4. A: Hematoxylin staining showing three histological different areas, ie, nest of Brunn (area 1), normal epithelium (area 2), and CIS (area 3), with corresponding hybridization images in B, C, and D, respectively. The CIS region was classified as tetrasomic for 7c, 9c, and 9p21, whereas the other areas were disomic. FISH with directly labeled probes for chromosomes 7, 9, and 9p21 are designated as green (7c), red (9c), and blue (9p21); numbers (eg, 2/2/2) indicate the classified copy numbers for the three probes, respectively. E–H: Case 16. E and G: p53-positive staining in two different CIS areas, with corresponding FISH images in F and H, respectively. In F an aneusomy for both chromosomes without loss of chromosome 9. Arrowhead indicates polyploid cell (high copy numbers for both chromosomes). In H loss of chromosome 9 is detected. FISH with probes for chromosomes 1 and 9 in a dual-color tyramide signal amplification detection system, designated as green (1c) and red (9c). Numbers (eg, 4/4) indicate the classified copy numbers for the two probes, respectively. I–K: Case 19. I: Hematoxylin staining of a synchronous lesion demonstrating detached cells. J: Cells with a disomy for the three loci (stromal site). K: Selective loss of chromosome 9 together with a homozygous deletion of 9p21. Probe set, colors, and numbers see A–D. L–N: Case 21. L: Hematoxylin staining of a synchronous papillary tumor showing different graded areas G2 (area 1), G3 (area 2), and corresponding hybridization images in M and N, respectively. The G2 area was classified as disomic for 1c and monosomic for 9c; the high grade as tetrasomic for 1c and a disomic for 9c. For probe set, colors, and numbers see E–H.
copy numbers equal to 1c and 7c, and those with losses of 9 in comparison to 1c and 7c.

The group without loss of chromosome 9, predominantly isolated CIS lesions with p53 accumulation (p53+), contained cells showing aneuploidy for all three chromosomes indicating an overall DNA aneuploidy. These data already suggested to us that accumulation of (mutated) p53 precedes loss of chromosome 9.

The second group, predominantly CIS with synchronous carcinomas, also showed an aneuploidy for chromosomes 1 and 7, but in these cases an evident imbalance with respect to chromosome 9 was detected, always indicating loss. A tendency toward development of metaphase sequence lesions was observed in the isolated CIS cases, whereas those with relative losses of chromosome 9 were at the same time associated with a synchronous pT1 carcinoma and showed an accumulation of p53. In contrast, lesions without p53 overexpression and harboring a homozygous deletion of 9p21 were observed in patients with concomitant pTa tumors.

The observed differential loss of chromosome 9 in CIS with and without concomitant invasive bladder carcinomas enlightens the controversy with respect to chromosome 9 losses that have been previously reported in CIS lesions. Spruck and colleagues identified only in 12% of the primary isolated CIS an allelic loss, whereas Rosin and colleagues found in 47% of CIS lesions a loss of heterozygosity for 9p and q. The latter study was however performed using a series of cases particularly selected from patients with synchronous carcinomas.

Our observation that in CIS lesions of patients with synchronous pT1 tumor areas with and without chromosome 9 losses have been recognized argues for the concept that a relative loss of chromosome 9 can occur during successive stages of tumor development toward an invasive carcinoma. Because both areas exhibit p53 overexpression these data also implicate that p53 mutations can occur before loss of chromosome 9, which confirms the suggested progression model of Jones and co-workers and Cordon-Cardo and colleagues. This might also be the case in half of the isolated CIS cases that showed overexpression of p53 and that showed a metachronous pT1 tumor within 2 years. These data support the view that mutant p53 should be regarded as the driver for genetic instability with the tendency to lose chromosome 9 copies. Thus, the presence of mutant p53, overall aneuploidy as well as loss of chromosome 9 are strong indicators for the presence or development of synchronous carcinomas.

Homozygous deletions of 9p21 were not observed in CIS lesions with synchronous pT1 tumors but were detected in all patients with synchronous high-grade pTaG3 tumors. Also, in two patients the low-grade lesions recognized in the tissue biopsy exhibited a homozygous deletion of 9p21 and a monosomy for chromosome 9. These lesions were further characterized by the absence of p53 accumulation (three of four cases). These observations are in agreement with previous studies that showed extensive losses on chromosome 9 in superficial tumors as compared to invasive carcinomas.

Our data suggest that these CIS lesions are the result of a process of polyploidization of diploid p53-negative cells harboring monosomy 9 and a homozygous deletion (HIZD) of 9p21. Furthermore, monosomies for chromosome 9 and loss of 9p21 are characteristic for low-grade papillary pTa carcinomas including their hyperplastic precursor lesions, and thus argues for an alternative genetic route. Although we should not over interpret the linkage between 9p21 HIZD and the occurrence of a synchronous pTaG2/G3 carcinoma, given the highly selected series studied here, we can anticipate that loss of chromosome 9, including a HIZD of 9p21, seems to have occurred at an early stage tumor development in these CIS lesions, other than the loss of chromosome 9 in the CIS lesions detected in the patients with a synchronous pT1 carcinoma. Despite the observed differences between the two groups, both CIS type of lesions can apparently also occur simultaneously. For example, in patient 21, a p53-positive CIS lesion with an aneuploidy for chromosomes 1, 7, and 9 (relative loss of chromosome 9), was identified adjacent to a p53-negative lesion that was rather the outcome of the process of aneuploidy after early loss of chromosome 9.

Genetic Model of Bladder Cancer Progression

Taking into account the histopathological observation that most invasive cancers of the bladder are derived from flat lesions known as CIS, and low-grade tumors are derived from simple hyperplasia, the current genetic models of bladder cancer progression and our results showing no loss of chromosome 9 in solitary CIS and p53 overexpression before chromosome 9 loss, we can summarize the early steps in tumor development in two distinct pathways of bladder carcinogenesis. In one of the routes early invasive pT1 lesions that reveal abnormalities of p53 originate from severe dysplasia/CIS without loss of chromosome 9 but reveal DNA aneuploidy. It needs further clarification whether or not dysplasia, defined as low-grade intraurothelial neoplasia, should be regarded as prestages of CIS (ie, high-grade intraurothe-
p53 should be regarded as the driver for genetic unstable (polyploid cells) in the latter. This may further trigger processes such as deregulation of adhesion-dependent apoptosis and clonal expansion. In the other route of bladder carcinogenesis pTa tumors, that are low grade, well differentiated, and usually papillary neoplasms, tend to recur but not progress. They are DNA diploid and exhibit early loss of the entire chromosome 9 or a homozygous deletion of 9p21 (p16/INK4a and p15/ INK4B), resulting in a growth advantage. It was remarkable that the grade transition G2 to G3 in the papillary pathway, which is an infrequent finding, was recognized three times as a synchronous carcinoma next to the CIS lesions. One may argue that the CIS lesion either evolved to a papillary carcinoma or that CIS lesions originate from the pTaG3 lesion. Our analyses and the fact that none of the pTaG3 lesions showed progression to pT1 is in favor of the latter hypothesis. The clinical prognostic implications of the combined identification of p53 and chromosome 9 alterations are currently under investigation.

**References**