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Frequent Genetic Alterations in Simple Urothelial Hyperplasias of the Bladder in Patients with Papillary Urothelial Carcinoma

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In order to understand the origin of bladder cancer, very early urothelial lesions must be investigated in addition to more advanced tumors. Tissue from 31 biopsies of 12 patients with urothelial hyperplasias and simultaneous or consecutive superficial papillary tumors were used to microdissect urothelium from 15-μm sections of biopsies. The biopsies were obtained with the recently developed highly sensitive diagnostic method of 5-aminolevulinic acid-induced fluorescence endoscopy (AFE). Besides flat and papillary urothelial neoplasms, the method of photodynamic diagnostics also detects simple urothelial hyperplasias as fluorescent positive lesions. In addition, 12 fluorescence-positive biopsies showing histologically normal urothelium were investigated. Fluorescence *in situ* hybridization was done using a dual color staining technique of biotinylated centromeric probes of chromosomes 9 and 17 and digoxigenin-labeled gene-specific P1 probes for chromosomes 9q22 (FACC), 9p21(p16/CDKI2), and 17p13(p53). Ten of 14 hyperplasias (70%) showed deletions of chromosome 9. In 7 out of 8 patients with genetic alterations in the hyperplasias the genetic change was also present in the papillary tumor. Six out of 12 samples of microdissected normal urothelium also showed genetic alterations on chromosome 9. Microdissection of urothelial lesions, obtained during AFE, has led to the first unequivocal documentation of genetic changes in urothelial lesions diagnosed as normal in histopathology. Thus, this technical approach is important to provide insight into the earliest molecular alterations in bladder carcinogenesis. ***(Am J Pathol 1999, 154:721–727)***

Precursor lesions of papillary neoplasms of the urinary bladder are not as well recognized as those in other organs such as uterine cervix and gastrointestinal tract, because the diagnosis of a symptomatic tumor is the primary clinical event, precluding the opportunity to study the genesis of these tumors.[1] The method of 5-aminolevulinic acid (5-ALA)-induced fluorescence endoscopy (AFE) provides a unique opportunity to study early preneoplastic urothelial lesions for insights into carcinogenesis in the bladder. AFE is based on the tumor cell-selective intracellular accumulation of protoporphyrin IX after intravesical administration of 5-ALA (see Fig. 1A).[2,3] In comparison to white light endoscopy, AFE detects a significantly higher rate of urothelial tumors, especially increasing detection of flat lesions as carcinoma *in situ* as well as small papillary tumors.[2] Tumor and dysplasia are detected not only in bladder urothelium but also in the gastrointestinal tract and in the lung.[4] Besides high sensitivity of tumor detection, about 30% of fluorescent mucosal areas in the urinary bladder are found to have a histologically benign diagnosis as squamous metaplasia, cystitis, normal urothelium, and urothelial hyperplasia. We hypothesized that AFE can identify histomorphologically inconspicuous urothelial alterations, some of which are already preneoplastic lesions. This hypothesis seems especially intriguing in light of recent work by Chatuverdi et al[5] showing deletions of part of chromosome 17 in histomorphologically normal urothelium in patients with muscle-infiltrating bladder cancer.

The finding of false positive lesions was used to start genetic analysis on histologically nonneoplastic urothelium. Biologically, the most interesting group seemed to be simple hyperplasia (Fig. 1, B and C). Flat urothelial hyperplasia is defined as cytologically unremarkable urothelium that is more than seven layers thick.[1] Koss first postulated the preneoplastic nature of urothelial hyperplasias without atypias, starting in 1975 that "urothelial hyperplasia frequently accompanies tumors of the bladder and is also undoubtedly the source of papillary neoplasia."[6]

To test this hypothesis, simple hyperplasia must be distinguished from flat or polypoid inflammatory lesions

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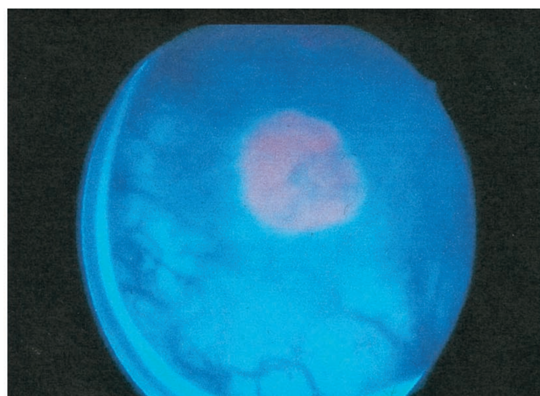

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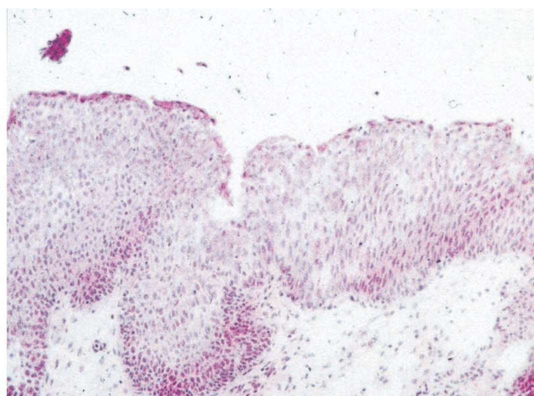

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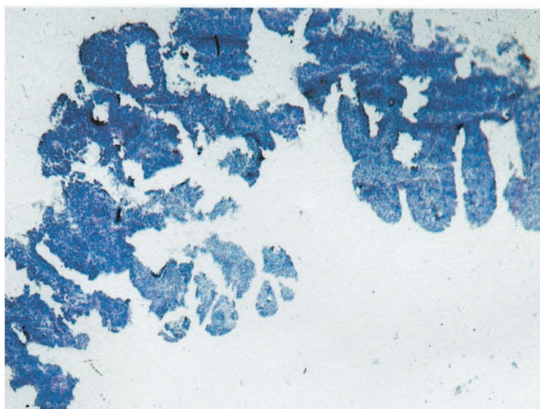

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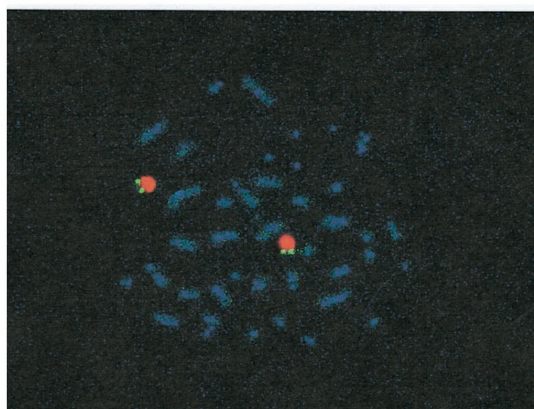

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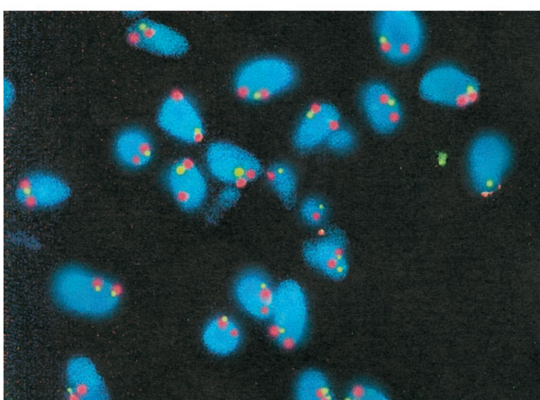

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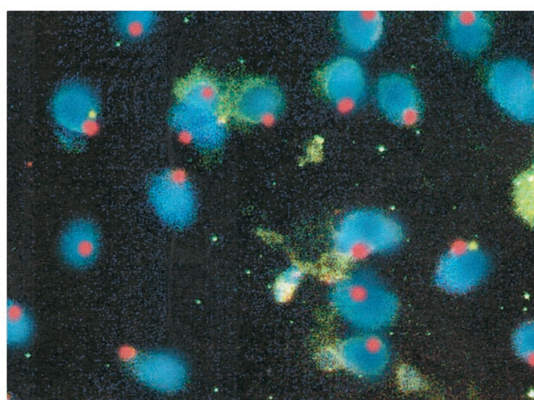

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Figure 1. A: ALA-induced fluorescence endoscopy of the bladder with a protoporphyrin IX red fluorescing flat lesion surrounded by blue autofluorescence (case 9). Histology of the red area revealed simple hyperplasia. B: Simple urothelial hyperplasia in the biopsy taken from the red fluorescing area in A. Thickened urothelium without papillary formations and disturbance of urothelial layering and inconspicuous nuclei are visible in a 5-μm frozen section stained with hematoxylin and eosin. Magnification, ×160. C: Methylene-blue stained 20-μm frozen section of a papillary tumor after microdissection. The papillary contours are clearly visible without significant amounts of residual stromal cells. The papillary tumor has been used as an example, since hyperplasias can be stripped more easily from the stromal compartment. Magnification, ×40. D: Fluorescence *in situ* hybridization using a centromeric probe for chromosome 9 and a gene-specific probe for chromosome 9p21 (p16) in metaphase spreads. Note the green signal on the short arm of chromosome 9. Magnification, ×1000. E: Fluorescence *in situ* hybridization using the probe for chromosome 9p21 (p16) in isolated nuclei of microdissected cells of the hyperplasia shown in A and B (case 8). The red signals are the centromeres of chromosome 9, whereas the green signals represent the gene-specific loci. There is a deletion of the 9p21 gene locus with two centromere signals and one gene-specific signal in the majority of the cells. Magnification, ×1000. F: Fluorescence *in situ* hybridization using the probe for chromosome 9p21 (p16) in isolated nuclei of microdissected cells of a hyperplasia (case 12). There is a homozygous deletion with one centromere signal and loss of the gene-specific signal in the majority of cells. Note rare nuclei with intact green gene-specific signal, which serves as an internal control for successful amplification. Magnification, ×1000.

Table 1. Histopathological and Clinical Data of 12 Patients with Fluorescence-Positive Urothelial Hyperplasias and Simultaneous or Consecutive Papillary Urothelial Tumors

		Hyperplasia		Papillary tumor		
Case	Age/Gender	Time point*	Localization	Stage/Grade†	Time point*	Localization
1	54/m		LW right	pTaG1	S	B
				pTaG1	C_1	LW left
2	49/m		PW	pTaG1	S	PW
				pTaG1	C_{20}	PW
3	71/m		LW right	pTaG1	S	LW left
4	67/m		B	pTaG1	S	D
5	56/f		LW right	pTaG2	S	B
6	60/m		PW	pTaG1	S	PW
7	52/m		LW left	pTaG1	C_1	LW left
				pTaG1	C_4	LW left
8	56/m		LW right	pT1G2	C_1	LW left
9	76/m		D	pTaG1	S	B
				pTaG1	C_8	T
10	34/m		LW left	pTaG1	S	D
		C_5	LW left			
11	59/m		D	pTaG1	S	D
		C_9	D			
12	64/m		LW left	pTaG1	S	PW
				pTaG1	C_3	LW left

m, male; f, female; S, simultaneous; C, $consecutive_{months}$; LW, lateral wall; PW, posterior wall; D, bladder dome; B, bladder base; T, trigonum.
*Time point of biopsy in relation to occurrence of the first hyperplasia.
†According to TNM classification.

with reactive urothelial hyperplasia, which have been grouped under cystitis in our study. We investigated 12 patients with simple urothelial hyperplasias and simultaneously or consecutively biopsied papillary tumors using dual-color fluorescence *in situ* hybridization (FISH). FISH is a powerful tool for visualizing quantitative genomic alterations in single cells. Key changes in bladder cancer development are deletions on both arms of chromosome 9 and p53 gene alterations, possibly representing alternative pathways to malignant progression.[7–9] Chromosome 9 deletions occur in 70% of all bladder cancers, whereas inactivation of the p53 tumor suppressor gene occurs in a high frequency in transitional carcinoma *in situ* and invasive bladder cancer.[10] There is evidence that chromosome 9 deletions are an early event in papillary bladder cancer with involvement of at least three different loci (9p21, 9q13-31, and 9q32-33).[11,12] Using gene-specific probes for 9q22 (Fanconi anemia complementation group C, or FACC),[13] 9p21 (CDKI2), and 17p13 (p53) and centromere probes for enumeration of chromosomes 17 and 9, we studied cells of 14 hyperplasias and 17 papillary tumors by FISH to detect deletions within these genomic regions. Furthermore, 12 AFE-positive biopsies of normal urothelium were investigated. In order to analyze urothelial cells without contaminating stromal cells, the urothelium was microdissected from the adjacent stromal cells. Ten of 14 simple hyperplasias showed evidence for genetic alterations involving chromosome 9, which were also found in the papillary tumors of these patients. Furthermore, 6 of 12 samples of normal urothelium showed chromosome 9 alterations. Using AFE-positive biopsies with normal histology and FISH after careful microdissection of the urothelial cells, we could show for the first time alterations of chromosome 9 in biopsies considered normal by histopathological examination.

Materials and Methods

Patient Material and Histopathological Diagnosis

Cystoscopy was performed after intravesical instillation of 5-ALA in patients participating in a clinical trial evaluating the photodynamic diagnosis of bladder cancer.[2] All patients gave written informed consent for the study. Biopsies were obtained from fluorescent lesions, immediately snap-frozen in the operating room, and shipped on dry ice. Histologic diagnosis was established on serial frozen sections stained with hematoxylin-eosin. Staging was performed according to Union Internationale Contre le Cancer[14] and grading according to the World Health Organization.[6] Twelve patients with 14 simple urothelial hyperplasias (two patients had two consecutive hyperplasias) and simultaneously ($n = 10$) or consecutively ($n = 7$) biopsied AFE-positive papillary superficial tumors (15 pTaG1, 1 pTaG2, and 1 pT1G2) were selected for genetic analyses (Table 1). Simple hyperplasia was diagnosed as a lesion with thickened urothelium (>7 layers) in serial sections, excluding cases with significant inflammatory infiltrate and edema in the adjacent stroma, as well as cases with urothelial atypia.[6] Samples were intentionally limited to flat urothelial hyperplasia (Figure 1A) because intraepithelial papillary lesions, especially papillary hyperplasia,[15] often could not be separated from a pTaG1 tumor when small branches of urothelium were found in serial sections. Four patients had lesions with identical locations in the bladder, whereas the locations differed in eight patients. Also, 12 AFE-positive biopsies of normal urothelium of seven patients with bladder cancer were examined. Histology of these biopsies showed neither dysplasia nor reactive inflammatory changes.

Microdissection and Cell Dissociation

From each frozen sample a 4-μm frozen section was stained with hematoxylin-eosin and the presence of tumor or hyperplasia was confirmed. Two consecutive 15-μm sections were stained with methylene blue for approximately 15 seconds. The tumor or hyperplasia was separated from stromal cells by microdissection with a needle (22G) under an inverted microscope (40× magnification). The microdissected probes contained at least 90% urothelial cells (Figure 1C). The cells were incubated in CT100 (citric acid/0.5% Tween) for 60–180 minutes at room temperature until cytoplasm of cells was dissolved. The cells were pelleted on silanized glass slides by standard microcentrifugation, fixed in freshly prepared methanol/acetic acid (3:1), air-dried, and stored at −20°C for up to 3 months.

DNA Probes and Probe Labeling

For counts of chromosomes 9 and 17, biotin-labeled centromeric probes (D9Z1 and D17Z1, Oncor, Gaithersburg, MD) were used. These probes were combined with P1 probes, obtained from the Lawrence Berkeley National Laboratory/University of California San Francisco Resource for Molecular Cytogenetics. These probes have a length of approximately 60–80 kb and are cloned in pAd10SacBII.[16] The following probes were used: RMC09P007 for chromosome 9p21 (CDKI2/p16 locus), RMC09P008 for chromosome 9q22 (FACC locus), and RMC17P078 for chromosome 17p13 (p53 gene locus). DNA was isolated with alkaline lysis[17] and labeled with digoxigenin-11-dUTP using standard nick translation protocols (Boehringer Mannheim, Mannheim, Germany).

Fluorescence in Situ *Hybridization*

FISH was performed as described by Sauter et al.[18,19] Briefly, cells on slides were denatured in 70% formamide/2× SSC, pH 7.0, at 75°C for 2.5 minutes. After dehydration in graded ethanol (70%, 80%, and 100% for 2 minutes each), samples were treated with proteinase K (Sigma, St. Louis, MO) for 7 minutes at 37°C, followed again by ethanol dehydration. Proteinase K concentration varied between 0.4 and 0.8 μg/ml, depending on tissue preservation and duration of slide storage. The hybridization mixture was denatured for 5 minutes at 75°C and subsequently reannealed for 40 minutes at 37°C. Ten microliters of hybridization mixture (20–30 ng gene-specific probe, 5–10 ng unlabeled sonicated human placental DNA (Sigma), 1 μl centromeric probe (Oncor) in 50% formamide, 10% dextran sulfate and 2× SSC, pH 7.0) were applied to each cytospin. Hybridization was overnight at 37°C. Metaphase spreads were used as controls to assure specificity of the probes (Figure 1D). Furthermore, for every hybridization cytospins of cultured urothelial cells (Urotsa, John Masters, University Hospital, London) without any alterations at the investigated gene loci were included to assure an estimation of hybridization efficiency. The probes were visualized by immunostaining in three steps: 10 μg/ml FITC-conjugated anti-digoxigenin (Boehringer Mannheim), 0.3 μg/ml FITC-conjugated anti-sheep IgG (Sigma), and 0.3 μg/ml Texas Red avidin (Vector, Burlingame, CA) (eg, Figure 1E). Counterstaining was performed with DAPI in Vectashield mounting medium (Vector).

Scoring of FISH Signals

Cells were selected for scoring with DAPI staining according to morphological criteria. Clearly distinguishable small lymphocytes were disregarded and all other cells were scored. Slides were analyzed if >75% of cells were interpretable. Copy numbers for centromeres and specific gene regions were counted in 200 cells, if possible (minimum 60 cells for hyperplasias and papillary tumors, 45 cells for normal urothelium). Only cells with nonoverlapping and intact nuclei were counted. Cells without any signal were disregarded. All hybridizations were evaluated independently by two of the investigators (A.H. and K.M.) and the mean of both counts was used. As a measure of deletion, the percentage of cells containing either one copy of centromere 9 or 17 or fewer gene-specific signals than centromeric signals (defined as percentage of deletion) was calculated for each hybridization. The average percentage of deletion of 10 hybridizations of a normal urothelial cell line (Urotsa) and 6 hybridizations of dissociated normal urothelium from patients without bladder cancer was less than 15 ± 5% for every probe. Because there was no normal tissue available from patients treated in exactly the same manner as the investigated ones, a tumor was considered deleted for a specific chromosomal locus if the percentage of deletion was >40% (equivalent 2× mean ± SD), a conservative evaluation of the results. Monosomy and homozygous deletion were defined as more than ⅔ of all deleted cells having either of these two alterations.

Results

Ten of 14 AFE-positive hyperplasias (71%) showed monosomies or partial deletions of chromosome 9 (Table 2 and Figure 1, E and F). There were four deletions at 9p21/p16 without involvement of chromosome 9q. In contrast, deletion at 9q22/FACC was found only once in combination with a normal chromosome 9p. In five hyperplasias, monosomy of chromosome 9 was found. One hyperplasia (case 12) showed a homozygous deletion of the p16 locus at 9p21 in addition to a monosomy 9. The discrepancy between the monosomy detected with the 9q probe in two consecutive hyperplasias and one papillary tumor in patient 10 and the hemizygous deletion detected with the probe for 9p in one of the hyperplasias and the papillary tumor can be explained by the arbitrary definition for monosomy with at least ⅔ of the deleted population of cells. In this case the percentage of all counted cells with monosomy (one centromere and one gene-specific signal) was 43% for the 9q and 27% for the 9p locus. However, there was a subpopulation of tumor cells with a hemizygous deletion at 9p21, whereas the monosomic population predominated in the hybridization

Table 2. Results of Fluorescence *in Situ* Hybridization with Gene Locus-Specific Probes for Chromosomes 9p, 9q, and 17p

Case	Hyperplasia			Papillary tumor		
	9p21(p16)	9q22(FACC)	17p13(p53)	9p21(p16)	9q22(FACC)	17p13(p53)
1	N	N	N	N	N	N
				N	N	N
2	N	N	N	N	N	N
				N	N	N
3	N	N	N	M	M	N
4	N	N	N	HD	M	N
5	D	N	N	D	N	N
6	D	N	N	D	D	N
7	D	N	N	M	M	N
				M	M	N
8	D	N	N	HD	M	N
9	N	D	N	D	M	N
				D	M	N
10	D	M	N	D	M	N
	M	M	N			
11	M	M	N	M	M	N
	M	M	N			
12	HD	M	D	M	M	N
				M	M	D

N, normal; D, hemizygous deletion; M, monosomy; HD, homozygous deletion.
For definitions of the different groups see Materials and Methods section.

for the 9q locus. The same results explain the discrepancy between both arms of chromosome 9 in the papillary tumors in case 9. The discrepancies between the results obtained by FISH with two different hybridizations for chromosome 9 could be due to heterogeneity of tumor cells, differing slightly in aliquots of nuclei prepared from one sample. In all 12 patients, FISH results documented fewer or identical genetic changes in hyperplasias compared to the papillary tumors in the same patient. In 6 of 12 patients, additional genetic alterations had accumulated in the papillary tumor (Table 2). Only 2 of 12 patients (17%) had no deletions of chromosome 9 in all investigated hyperplasias and tumors. There was only one hyperplasia with a deletion of the p53 locus (case 12), which was also found in one of two consecutive papillary carcinomas. No other deletions of p53 were detected in any of the investigated hyperplasias and papillary tumors. Localization of hyperplasias in relation to the papillary tumor was found more often in a different area of the bladder wall than in the same area (Table 1). Thus, it was unlikely that only marginal areas of the papillary tumor were investigated.

Investigations were extended to 12 fluorescence-positive biopsies from 6 bladder tumor patients with the biopsy diagnosis of normal urothelium. Deletions of chromosome 9 were found in 6 of 12 (50%) of these biopsies (Table 3). Although four patients showed no deletions on chromosome 9 in the normal urothelium, one patient presented with deletions of chromosome 9 in 4 out of 5 biopsies (case 17). In another patient, both samples of normal urothelium had deletions of chromosome 9 (case 18). Categorization of hemizygous deletion, monosomy, and homozygous deletion was not done in these patients because of the small number of nuclei obtained (45–85 cells) due to the small size of the urothelial areas which had been microdissected. There were no deletions on chromosome 17 in any of the investigated normal urothelial biopsies.

Discussion

For the first time, urothelial lesions which are considered benign were investigated for genetic alterations using FISH in patients with papillary bladder cancer. The methods used in this study are novel and enabled us to detect genetic alterations at a high frequency for several reasons. First, biopsies were obtained by AFE, a method that detects a higher frequency of flat neoplastic lesions.[2,3] Second, precise microdissection of the urothelial areas in the biopsies ensured the investigation of a homogenous cell population with low contamination by fibroblasts and inflammatory cells. Third, FISH using a dual-color label-

Table 3. Results of Fluorescence *in Situ* Hybridization Investigating Fluorescence-Positive Normal Urothelium with Gene Locus-Specific Probes for Chromosomes 9p, 9q, and 17p

Case	9p21(p16)	9q22(FACC)	17p13(p53)
1	N	N	N
2	N	N	N
3	N	N	N
	N	N	N
4	N	N	N
5	N	n.a.	N
	D	N	N
	D	D	N
	D	N	N
	D	D	N
6	D	D	N
	D	D	N

For definition of a deletion see Materials and Methods section.
N, normal; D, hemizygous deletion; n.a., not available.

ing technique with centromeric and gene-specific probes enabled us to investigate specific genetic alterations known to occur in early bladder cancer. Fourth, investigation of urothelial hyperplasias and papillary tumors in the same patients allowed a comparison of the genetic alterations in both lesions.

With these techniques, genetic alterations similar to papillary urothelial carcinomas were found in simple urothelial hyperplasias. Deletions of genetic material of chromosome 9 can be demonstrated in more than 70% of AFE-positive hyperplasias. The frequency of chromosome 9 deletions in the papillary tumors of these patients was 76%. The majority of these tumors (64%) showed a monosomy 9. In contrast, deletions of the p53 locus were rare in both simple hyperplasias and papillary tumors. These results are comparable with other studies using both FISH and loss of heterozygosity analyses for detection of chromosome 9 and p53 deletions in bladder cancer.[7,9,11,12,18–21] Furthermore, in two of six investigated patients, chromosome 9 deletions were also detected in biopsies of normal urothelium. FISH results with the gene locus probe of p53 were counted normal in these biopsies.

It has recently been hypothesized that chromosome 9 alterations are an early event in the development of papillary bladder cancer.[7,9,11] The finding that simple urothelial hyperplasias in patients with papillary bladder cancer have the same frequency of chromosome 9 alterations and are clonally related to the corresponding tumors provides strong evidence for this hypothesis. At least a subset of urothelial hyperplasias seems to represent preneoplastic lesions showing the same molecular alterations as related tumors. The increase of genetic alterations in papillary tumors *versus* hyperplasias in 50% of the patients may be interpreted as the existence of subclones, which may have accumulated genetic alterations progressively. Chatuverdi et al[5] showed, in a study using a genetic-histological mapping approach of the entire bladder in bladder cancer patients, that there are deletions at several loci of chromosome 17 detected by loss of heterozygosity analysis in areas of urothelium which are considered benign by conventional histology. These data, together with the results of our study, support the hypothesis that many areas of the bladder are genetically altered in patients with bladder cancer. Genetic investigation of areas with different histology and large series of normal urothelial biopsies in bladder cancer patients could prove this hypothesis and provide insights into the first steps of bladder carcinogenesis. The methodology used in this study is considered of significant help in this regard.

Interestingly, in 1/3 of the investigated hyperplasias, deletions on chromosome 9p21/p16 seem to precede the deletion on chromosome 9q, whereas in only one case was a 9q alteration the earlier event. This is in contrast to data of Simoneau et al[11] who found deletions in 9q without alteration of 9p in 4 of 37 papillary tumors. However, 11 cases in 110 transitional cell carcinomas (10%) having 9p deletions without 9q alterations are reported in the literature.[22] In a series of multifocal papillary urothelial cancers, 9p21 deletions without alterations on 9q22 were found in an additional 8 tumors using the same methodology (data not shown). Recently, loss of heterozygosity studies demonstrated that inactivation of multiple tumor suppressors on chromosome 9 may occur during bladder cancer development.[11,12] One of the two defined loci on chromosome 9q is located on 9q13-31 and is covered by the FACC probe for 9q22 used in this study. The other locus was mapped to 9q32-33[12] and a novel gene, DBCCR1, showing frequent methylation-based silencing, was recently cloned in this region.[23] Small subchromosomal deletions in this region would have been missed in this study. However, we do not assume to have underestimated the frequency of deletions at 9q because the majority of deletions involve the whole chromosome arm. Larger sample numbers need to be analyzed to clarify which of the deletions on chromosome 9 occurs first during tumorigenesis.

Thorough molecular investigation of AFE-positive simple hyperplasias using microdissection of these lesions, whole genome amplification by primer extension preamplification-polymerase chain reaction,[24,25] and subsequent deletion mapping of chromosome 9 and other regions, combined with comparative genomic hybridization[26] and FISH, will provide a broader picture of the molecular alterations already present in these lesions. The fact that in patients with 9p21 deletion as single genetic event in 3 of 4 corresponding papillary tumors a deletion of chromosome 9q or monosomy 9 was detectable, provides evidence that AFE-positive hyperplasias could be indeed an early neoplastic lesion in the urinary bladder and a good source of material to reveal the earliest molecular alterations in bladder carcinogenesis. In most patients in this study, the locations of hyperplasias and papillary tumors within the urinary bladder differed. This finding strengthens the argument that the simple hyperplasia may be a precursor lesion for bladder cancer.

Our findings may further support the hypothesis that AFE enables more complete excision of bladder lesions, including lesions that are invisible in white light endoscopy. While genetic alterations of simple urothelial hyperplasias and normal urothelium have been documented for the first time with this study, investigations of large series of patients with AFE-positive and -negative hyperplasias as well as normal urothelium and clinical follow-up of these patients are necessary to completely define the importance of urothelial hyperplasias in the multistep process of tumorigenesis in the urinary bladder.

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References

1. Amin MB, Young RH: Intraepithelial lesions of the urinary bladder with a discussion of the histogenesis of urothelial neoplasia. Semin Diagn Pathol 1997, 14:84–97
2. Kriegmair M, Baumgartner R, Knuchel R, Stepp H, Hofstadter F,

Hofstetter A: Detection of early bladder cancer by 5-aminolevulinic acid induced porphyrin fluorescence [see comments]. J Urol 1996, 155:105–109
3. Steinbach P, Kriegmair M, Baumgartner R, Hofstadter F, Knuchel R: Intravesical instillation of 5-aminolevulinic acid: the fluorescent metabolite is limited to urothelial cells. Urology 1994, 44:676–668
4. Peng Q, Warloe T, Berg K, Moan J, Kongshaug M, Giercksky KE, Nesland JM: 5-Aminolevulinic acid-based photodynamic therapy: clinical research and future challenges. Cancer 1997, 79:2282–2308
5. Chatuverdi V, Hodges S, Johnston D, Ro JY, Logothetis C, von Eschenbach AC, Batsakis JG, Czerniak B: Superimposed histologic and genetic mapping of chromosome 17 alterations in human urinary bladder neoplasia. Oncogene 1997, 14:2059–2070
6. Koss LG: Tumors of the urinary bladder. Atlas of Tumor Pathology. Vol. 16 (2nd series, fascicle 11). Washington, DC, Armed Forces Institute of Pathology, 1975
7. Spruck CH, Ohneseit PF, Gonzalez Zulueta M, Esrig D, Miyao N, Tsai YC, Lerner SP, Schmutte C, Yang AS, Cote R, Dubeau L, Nichols PW, Herrman GG, Steven K, Horn T, Skinner DG, Jones PA: Two molecular pathways to transitional cell carcinoma of the bladder. Cancer Res 1994, 54:784–788
8. Dalbagni G, Presti J, Reuter V, Fair WR, Cordon Cardo C: Genetic alterations in bladder cancer [see comments]. Lancet 1993, 342: 469–471
9. Ruppert JM, Tokino K, Sidransky D: Evidence for two bladder cancer suppressor loci on human chromosome 9. Cancer Res 1993, 53: 5093–5095
10. Cordon Cardo C, Dalbagni G, Sarkis AS, Reuter VE: Genetic alterations associated with bladder cancer. Important Adv Oncol 1994, 1:71–83
11. Simoneau AR, Spruck CH, Gonzalez Zulueta M, Gonzalgo ML, Chan MF, Tsai YC, Dean M, Steven K, Horn T, Jones PA: Evidence for two tumor suppressor loci associated with proximal chromosome 9p to q and distal chromosome 9q in bladder cancer and the initial screening for GAS1 and PTC mutations. Cancer Res 1996, 56:5039–5043
12. Habuchi T, Yoshida O, Knowles MA: A novel candidate tumour suppressor locus at 9q32–33 in bladder cancer: localization of the candidate region within a single 840 kb YAC. Hum Mol Genet 1997, 6:913–919
13. Strathdee CA, Duncan AM, Buchwald M: Evidence for at least four Fanconi anaemia genes including FACC on chromosome 9. Nat Genet 1992, 1:196–198
14. Sobin LH and Wittekind: TNM Classification of malignant tumours. Fifth Edition. New York, Weinheim, Wiley-Liss, 1997
15. Taylor DC, Bhagavan BS, Larsen MP, Cox JA, Epstein JI: Papillary urothelial hyperplasia. A precursor to papillary neoplasms. Am J Surg Path 1996, 20:1481–1488
16. Shepherd NS, Pfrogner BD, Coulby JN, Ackerman SL, Vaidyanathan G, Sauer RH, Balkenhol TC, Sternberg N: Preparation and screening of an arrayed human genomic library generated with the P1 cloning system. Proc Natl Acad Sci USA 1994, 91:2629–2633
17. Lee S, Rasheed S: A simple procedure for maximum yield of high-quality plasmid DNA. Biotechniques 1990, 9:676–679
18. Sauter G, Deng G, Moch H, Kerschmann R, Matsumura K, De Vries S, George T, Fuentes J, Carroll P, Mihatsch MJ, Waldman FM: Physical deletion of the p53 gene in bladder cancer: detection by fluorescence in situ hybridization. Am J Pathol 1994, 144:756–766
19. Sauter G, Moch H, Carroll P, Kerschmann R, Mihatsch MJ, Waldman FM: Chromosome-9 loss detected by fluorescence in situ hybridization in bladder cancer. Int J Cancer 1995, 64:99–103
20. Miyao N, Tsai YC, Lerner SP, Olumi AF, Spruck CH, 3d, Gonzalez Zulueta M, Nichols PW, Skinner DG, Jones PA: Role of chromosome 9 in human bladder cancer. Cancer Res 1993, 53:4066–4070
21. Linnenbach AJ, Pressler LB, Seng BA, Kimmel BS, Tomaszewski JE, Malkowicz SB: Characterization of chromosome 9 deletions in transitional cell carcinoma by microsatellite assay. Hum Mol Genet 1993, 2:1407–1411
22. Gonzalez Zulueta M, Shibata A, Ohneseit PF, Spruck CH, Busch C, Shamaa M, El Baz M, Nichols PW, Gonzalgo ML, Malmstrom PU, Jones PA: High frequency of chromosome 9p allelic loss and CDKN2 tumor suppressor gene alterations in squamous cell carcinoma of the bladder (published erratum appears in J Natl Cancer Inst 1995, 87:1807). J Natl Cancer Inst 1995, 87:1383–1393
23. Habuchi T, Luscombe M, Elder PA, Knowles MA: Structure and methylation-based silencing of a gene (DBCCR1) within a candidate bladder cancer tumor suppressor region at 9q32–q33. Genomics 1998, 48:277–288
24. Zhang L, Cui X, Schmitt K, Hubert R, Navidi W, Arnheim N: Whole genome amplification from a single cell: implications for genetic analysis. Proc Natl Acad Sci USA 1992, 89:5847–5851
25. Dietmaier W, Hartmann A, Wallinger S, Heinmoeller E, Kerner T, Jauch KW, Hofstaedter F, Rueschoff J: Multiple mutation analysis in single tumor cells enabled by improved whole genome amplification. Am J Pathol 1999, 154:83–95
26. Richter J, Jiang F, Gorog JP, Sartorius G, Egenter C, Gasser TC, Moch H, Mihatsch MJ, Sauter G: Marked genetic differences between stage pTa and stage pT1 papillary bladder cancer detected by comparative genomic hybridization. Cancer Res 1997, 57:2860–2864