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Cytogenetic Analysis of Multifocal Bladder Cancer Supports a Monoclonal Origin and Intraepithelial Spread of Tumor Cells[1]

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ABSTRACT

Bladder cancer is often characterized by a multifocal growth pattern. This observation has given rise to the hypothesis of "field cancerization," predicting a polyclonal origin of multiple tumors rising from an area of independently transformed mucosa cells. On the other hand, genetic studies suggested a monoclonal origin. To address these contradictory hypotheses, we performed comparative genomic hybridization (CGH) on 32 tumors originating from six bladder cystectomy specimens. All tumors derived from the same patient showed a set of 7–13 identical chromosomal aberrations and additional individual alterations. Most striking were the findings of 17p losses in all (32 of 32) tumors of the six cystectomy specimens and 20p gains in all tumors of four bladders, as well as an unexpected high number of chromosomal changes (20.4 alterations per tumor on average). To clarify a possible role of the *TP53* tumor suppressor gene on 17p13, we applied immunohistochemistry and sequence analysis on the tumors and additional 52 mucosa samples. Identical *TP53* mutations and protein overexpression was found in individual tumors only as well as in mucosa samples from continuous areas. Our results not only provide further evidence for a monoclonal origin of multifocal bladder cancer but also point at intraepithelial migration of tumor cells carrying specific chromosomal aberrations.

INTRODUCTION

About one-third of all bladder cancers occur as a multifocal disease forming several tumors simultaneously at different sites of the bladder wall. During the last years, different concepts have been assumed to explain this phenomenon. One is the field defect hypothesis, according to which individual cells of the bladder urothelium are primed to undergo transformation because of environmental mutagens, consequently leading to the development of independent multiclonal tumors. This theory is mainly based on morphological and immunohistochemical mapping studies demonstrating areas of modified cells adjacent to the sites of the tumors (1, 2). In contrast, genetic studies have given evidence for a monoclonal origin. Sidransky *et al.* (3) showed that all tumors of a single patient with multifocal disease revealed the same X chromosome inactivation pattern. Additionally, mostly the same patterns of loss of heterozygosity (4) as well as identical *TP53* mutations (5) were detected. Most investigators, therefore, concluded that an intraluminal seeding or intraepithelial migration of cells originating from a single primary tumor might be responsible for multifocal tumor occurrence.

Most of the previous studies on multiple bladder cancer have been focused on few specific genetic changes (4, 6–10). However, bladder cancer is characterized by highly complex chromosomal changes affecting numerous chromosomal loci. Recently, a cytogenetic study provided a more complex overview about chromosomal changes in six cases of multifocal bladder cancer, showing a highly similar pattern of genetic changes in distinct tumors (11). However, a systematic analysis of both multifocal tumors and the surrounding urothelium to find clues for the mechanism of multifocal tumor development has not been performed yet.

We analyzed a set of 32 multifocal bladder carcinomas and 52 tissue samples from macroscopically uninvolved urothelium originating from six cystectomy specimens. In our study, we used CGH[3] that detects all DNA gains and losses present in a tissue sample to search for typical patterns of chromosomal aberrations in multiple bladder cancer. Our data not only provide further evidence for a monoclonal origin of multifocal bladder cancer but also point at intraepithelial migration of tumor cells carrying specific chromosomal aberrations.

MATERIALS AND METHODS

Materials. Cystectomy specimens of six bladder cancer patients (five males and one female) with multifocal disease were investigated. Samples were taken from 32 different tumors of the bladder specimens containing three, four, five, five, six, and nine tumors, respectively. Additionally, a total of 52 tissue samples were taken from sites of macroscopically uninvolved urothelium surrounding the tumors as well as from distant sites. The histological classification of each sample is given in Table 1.

DNA Isolation. Genomic DNA of the 32 unfixed tumor samples was prepared following standard DNA extraction procedures. If necessary, laser microdissection (PALM) was carried out on 10-μm sections to assure a tumor cell content of at least 80%.

CGH. All 32 tumors were investigated by CGH. CGH analysis and the criteria for the evaluation of copy number changes have been described elsewhere (12, 13). CGH profile shifts were rated as gains and losses if they at least reached the 1.25 and 0.75 thresholds. The Cytovision 3.1 software package (Applied Imaging International, Ltd.) was used for digital image analysis and subsequent karyotyping.

TP53 IHC Analysis. Sections (4 μm) of all formalin-fixed tissue samples of the 32 tumors and the 52 mucosa samples were investigated for *TP53* accumulation by immunostaining. The procedure and the antibodies used have been described elsewhere (14). Scoring was performed to the following criteria: − (negative), cells completely lacking nuclear staining; (+) (very weak), weak nuclear staining in widely scattered and <10% of cells; + (weak), weak nuclear staining or strong immunostaining in widely scattered and <10% of cells; ++ (moderate), strong immunostaining in 10–50% of cells or moderate staining in >50% of cells; +++ (strong), strong immunostaining in >50% of cells.

TP53 Mutation Analysis. All tumors of cases 3227, 3253, and 3312 (n = 15) were analyzed for *TP53* gene mutations because they showed at least moderate TP53 immunostaining (see "Results"). In addition, the TP53 IHC-positive mucosa sample IX of case 3253 was analyzed. Sequencing of the remaining IHC-positive mucosa samples was not performed because the content of IHC-positive cells was too small to allow the detection of a potential mutation. Mutation analysis was performed by solid phase sequencing of single-stranded PCR products from exons 5 to 8 of the *TP53*

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[3] The abbreviations used are: CGH, comparative genomic hybridization; IHC, immunohistochemistry.

Table 1 *Histological diagnosis and TP53 immunohistochemistry results of tissue samples taken from six cystectomy specimens*

TP53 scoring is explained in detail in "Materials and Methods."

Case no.	Sample	Diagnosis	TP53 score
3069	I	pTis[a]	(+)
	III	pTis	(+)
	IV	Tumor A (pT_a G3)	(+)
	V	Tumor B (pT_a G3)	(+)
	VI	Tumor C (pT_a G3)	+
	VII	Tumor D (pT_1 G3)	+
	VIII	Tumor E (pT_1 G3)	+
	XI	Normal urothelium	−
	XII	pTis	−
	XIV	pTis	(+)
	XV	pTis	−
	XVI	Dysplasia	(+)
3211	I	Tumor A (pT_3 G3)	−
	II	Tumor B (pT_1 G3)	(+)
	III	Tumor C (pT_2 G3)	(+)
	IV	Tumor D (pT_2 G3)	(+)
	V	Tumor E (pT_1 G3)	−
	VI	Tumor F (pT_1 G3)	−
	VII	Tumor G (pT_1 G3)	+
	VIII	Tumor H (pT_1 G3)	+
	IX	Tumor I (pT_1 G3)	+
	X	pTis	+
	XII	Tumor (pT_1 G3)	(+)
	XIII	pTis	−
	XIV	Dysplasia	−
	XV	pTis	−
3227	I	Tumor A (pT_3 G3)	++
	II	Tumor B (pT_2 G3)	+++
	III	Tumor C (pT_2 G3)	+
	IV	Tumor D (pT_2 G3)	+
	V	Tumor E (pT_2 G3)	(+)
	VIII	Tumor (pT_2 G3)	+
	X	Tumor (pT_2 G3)	(+)
	XI	Dysplasia	−
	XII	Dysplasia	−
	XIII	Normal urothelium	−
3253	I	Tumor A (pT_1 G3)	+++
	II	Tumor B (pT_a G3)	++
	III	Tumor C (pT_a G3)	++
	IV	Tumor D (pT_1 G3)	−
	VII	Dysplasia	−
	VIII	Normal urothelium	(+)
	IX	Dysplasia	++
	X	Normal urothelium	−
	XI	Tumor (pT_1 G3)	++
	XIV	pTis	++
	XV	Dysplasia	(+)
	XVI	Dysplasia	−
3287	I	Tumor A (pT_3 G3)	(+)
	II	Tumor B (pT_a G3)	+
	III	Tumor C (pT_1 G3)	+
	IV	pTis	(+)
	V	pTis	+
	VI	Tumor (pT_3 G3)	(+)
	VIII	Dysplasia	−
	IX	Dysplasia	−
	X	Dysplasia	−
3312	I	Tumor A (pT_a G3)	−
	II	Tumor (pT_a G3)	−
	III	Tumor B (pT_2 G3)	++
	IV	Tumor C (pT_1 G3)	++
	V	Tumor D (pT_2 G3)	++
	VI	Tumor E (pT_3 G3)	++
	VII	Tumor F (pT_4 G3)	++
	VIII	pTis	+
	IX	Dysplasia	−
	X	Dysplasia	++
	XI	Normal urothelium	++
	XII	Dysplasia	++
	XIII	Dysplasia	−
	XIV	Normal urothelium	(+)

[a] pTis, flat carcinoma *in situ*; pTa, papillary noninvasive carcinoma; pT1, invasion of the suburothelial stroma; pT3–pT4, invasion of the muscular bladder wall; G1–G3, histological grades 1–3.

gene, which are known to harbor ~80% of all mutations (15). The procedure was performed as described before (16). Gel electrophoresis, data collection, and analysis was performed on an automated laser fluorescence sequencer (A.L.F.; Pharmacia).

RESULTS

CGH. CGH ratio profiles were generated from DNA samples of each macroscopically identified tumor. All tumors derived from the same patient shared a set of 7–13 identical alterations (hereafter referred to as "basic changes"). Additional genetic changes were found in individual tumors or subsets of the tumors. On average, all 32 tumors investigated showed 20.4 chromosomal changes (range, 6–33).

The most frequent change was a loss of chromosome 17p material that was present as a basic change in 32 of 32 tumors (100%) of the six bladder specimens. Gains involving 20p affected all tumors of four cases. Interestingly, particular aberrations appeared frequently in combination as basic changes, whereas others were seen in the course of progression only (Table 2).

Following the theory of an accumulation of genetic changes during tumor development and progression (17), we developed cytogenetic pedigrees reflecting the accumulation of chromosomal aberrations for each case of multifocal bladder cancer (Fig. 1). Starting from a hypothetical precursor cell population characterized by the set of basic changes (X_0 in Fig. 1), additional clones (X_1–X_n) were identified based upon the highest number of identical chromosomal aberrations in addition to the precursor clone (Fig. 2).

***TP53* Immunohistochemical Analysis.** All 32 tumors and 39 of 52 mucosa samples were successfully analyzed by IHC. The results are given in detail in Table 1. A moderate or strong positive immunostaining [++ or +++ (positive)] was detected in individual samples of cases 3227, 3253, and 3312. In case 3227, only tumors A and B showed positive staining but none of the remaining samples. In cases 3253 and 3227, the positive samples were located in a contiguous area. In case 3253, this area included tumors A, B, and C as well as mucosa samples IX (dysplasia), XI (pT_1 tumor), and XIV (carcinoma *in situ*; Fig. 3). In case 3312, positive staining was found in tumors B, C, D, E, and F and mucosa samples X (dysplasia), XI (normal urothelium), and XII (dysplasia; Fig. 4).

***TP53* Mutation Analysis.** The findings of 17p deletions in all 32 tumors and positive TP53 immunostaining in 15 tumors investi-

Table 2 *Summary of the most frequent genetic changes in the tumors of the six bladder specimens investigated by CGH*

Aberrations occurring solely or predominantly (in 50–100% of cases) as basic changes are likely to be acquired early in multifocal tumor evolution, whereas those affecting a subset or single tumors only may represent late changes rather.

Chromosomal aberration	Aberration is a basic change (% of cases)	Cystectomy specimen					
		3069	3253	3227	3287	3312	3211
−17p	100	■[a]	■	■	■	■	■
+20p	100		■	■	■	■	
−9p	75	■	■	■			□
−9q	66	□	■	□	■	■	■
+2q34–qter	60	■	□		■	■	□
+12q14–q21	60	■	□	□	■	■	
+1q22–q25	50	■	□	□	■	□	■
−8p22–pter	50	■	□	■	■	□	
−5q31–qter	50	□		□	■	■	
+17q	50	□	■	■	□		
+11q14	25	■	□			□	□
−21q	25	□		□		□	■
−5q13–q14	20	□		□	■	□	□
+8q22	20		□	□	■	□	□
+10p	20	■	□	□	□	□	
−10q22–qter	20	■	□	□	□	□	
−11p	17	□	□	□	□	■	□
+3q24–q26	0	□	□	□	□		□
+2p21–cen	0	□		□	□	□	□
+3p26	0	□	□		□	□	□
+5p13–p14	0	□		□	□	□	□
+6pter–p22	0	□		□	□	□	□
−22q	0	□			□	□	□

[a] ■, change affects all tumors of the belonging cystectomy specimen (basic change); □, change affects a subset or single tumors only.

gated suggested an involvement of the *TP53* tumor suppressor gene. Sequencing of *TP53* of the IHC-positive tumors revealed point mutations in individual tumors of two bladder specimens. In case 3253, a transition from guanine to adenine at exon 8 codon 269 was found in tumors A, B, and C that resulted in the change of GAG (glutamic acid) to AAG (lysine). Tumor D showed the wild-type sequence. The TP53 IHC-positive mucosa sample (sample IX) revealed a mixed population of tumor cells carrying the known mutation and *TP53* wild-type mucosa cells (Fig. 3). In case 3227, also a transition from guanine to adenine occurred but affected exon 5 codon 179. It was present in tumors A and B but not in tumors C, D, and E (data not shown). In both cases, the mutations resulted in a moderate or strong TP53 immunostaining (++ or +++). None of the tumors without mutations was IHC positive. The tumors of case 3227 did not show mutations within the analyzed exons.

DISCUSSION

In our present study, we demonstrated a close genetic relationship between all tumors of a particular cystectomy specimen in six cases of multifocal bladder cancer. Comparison of the CGH ratio profiles obtained from the tumors belonging to the same case enabled us to elaborate individual cytogenetic pedigrees portraying the accumulation of chromosomal aberrations during multiple bladder cancer progression.

In our sets of tumors, between 7 and 13 identical chromosomal aberrations were found in each case as well as identical *TP53* mutations in two cases, indicating a clonal origin of the tumors. This is in concordance with previous studies that showed clonality by X chromosome inactivation patterns, genetically closely related or identical tumors, or *TP53* mutations in patients with multiple or recurrent tumors (3, 7, 10, 11).

The sequence of genomic changes acquired during progression of the tumors was highly individual and complex in each case. It can be assumed that early aberrations frequently affect all tumors of a case. Potentially early changes in this study include alterations such as −17p, +20p, −9p, −9q, +2q34–qter, +12q14–q21, +1q22–q25, −8p22–pter, −5q31–qter, and +17q. Subsequent tumor progression may be characterized by the accumulation of changes like +11q14, −21q, −5q13–q14, +8q22, +10p, −10q22–qter, and −11p, which occurred only rarely in all tumors of a case. Alterations appearing in particular tumors only, such as +3q24–q26, +2p21–cen, +3p26, +5p13–p14, +6pter–p22, and −22q, are likely to be very late changes. Under this assumption, the order of chromosomal aberrations outlined in Table 2 (from top to bottom) represents a hypothetical, unified sequence of frequent genetic changes acquired during evolution of multiple bladder cancer.

The majority of chromosomal changes detected in our study are commonly found in singular bladder cancer as well (12, 18–22). Some of them have been linked to special tumor properties, *e.g.,* tumor initiation (−9p, −9q; Refs. 12, 20, and 23), papillary growth (−9q; Ref. 21), high-grade or invasive phenotype (−8p, −11p, −17p; Refs. 12, 21, 22, and 24), or are suggested to represent late events (+5p, −5q; Refs. 21 and 22). Numerous genes that are known to play a role for tumor development may be affected by these alterations, *e.g., TP53* (17p13), *MDM2* (12q13–q15), and *PMS1* (2q34; apoptosis and maintenance of genomic stability), *CDK4* (12q13–q15), *CDK7* (2p15–cen), *CDKN2A* and *CDKN2B* (9p21; cell cycle regulation), or *DBCCR1* at 9q34 (tumor initiation). However, the findings of 17p losses in all tumors of all bladder specimens, 20p gains in all tumors of four bladder specimens, and the high number of aberrations per tumor are strikingly different from previous reports. In this regard, it is important to note that the majority of tumors investigated were late-stage, advanced carcinomas, because otherwise no cystectomy specimens would have been available. This means that our set of tumors is not entirely representative for typical multiple bladder carcinomas that are usually noninvasive.

The average number of aberrations per tumor (20.4) was remarkably higher as reported for pT_{2-4} (7.9 aberrations) or grade 3 carcinomas (7.8 aberrations) by means of CGH (21), suggesting an exceptional high degree of genomic instability in multifocal bladder cancer. However, it cannot be ruled out that the high number of chromosomal aberrations might be attributable to the preselection of extremely advanced tumors. Another point is that most of the previous CGH analyses have been performed on DNA obtained from formalin-fixed tissue samples. The direct comparison with CGH results from fresh tissue and may be problematic because shifts of the CGH profile are usually stronger in this case and might account for the larger number of profile shifts passing the thresholds. Nevertheless, it has been reported that mere reduction in TP53 levels may be sufficient to promote tumorigenesis (25). Loss of 17p that was present in all of our tumors may consequently account for a reduced activity of TP53 downstream mechanisms like induction of apoptosis, cell cycle arrest, and prevention of chromosomal instability, providing an alternative explanation for the high number of chromosomal defects.

However, the 100% rate of 17p losses must be interpreted with care because of the small number of cases. Nevertheless, it is remarkably higher than reported in non-multifocal bladder cancer by means of allelic loss (30–70%; Refs. 3 and 26–29) or CGH (up to 28%; Refs. 12, 20, and 21) and suggests a possible involvement of the *TP53* tumor suppressor gene. Yet, in our set of tumors, *TP53* mutations were detected in two of six cases only. Consequently, loss of 17p might be attributable to advanced tumor stage and grade rather than reflecting a characteristic alteration in multiple bladder cancer. On the other hand, it can be hypothesized that *TP53* may not be the target gene responsible for multiple tumor development. There might be one or more currently unknown genes located on 17p that may exert an influence on multifocality, *e.g.,* by inhibiting cell migration capabilities in healthy urothelium. A similar effect may be induced by gains involving chromosome 20p that appeared as a basic change in four of six cases (66%). This finding was surprising, because in non-multifocal bladder cancer, this alteration has been described rather rarely (9%; Ref. 21), arguing for a certain role of genes located on 20p for multiple bladder cancer.

Interestingly, cytogenetically closely related tumors revealed a close spatial relationship in this study. This raises the question on the mechanism of tumor cell spread leading to multifocal bladder cancer. To address this aspect we applied IHC for the detection of cells accumulating the TP53 protein in tumors and normal urothelium located between the tumors.

Notably, weak immunostaining (+) was found in most tumor samples of all cases but did not indicate a *TP53* gene mutation. Nuclear accumulation of TP53 can occur for several reasons besides intronic mutations, *e.g.,* overexpression of wild-type TP53 (30), interactions of TP53 protein with other intracellular or viral proteins (31, 32), or extensive posttranslational modification of wild-type TP53 (33). *TP53* gene mutations were solely detected in samples showing a moderate or a strong (++ or +++) immunostaining in our study. For this reason, analysis of IHC data to search for *TP53* mutated cells in the urothelium was restricted to those cases showing moderate or strong (++ or +++) immunostaining.

In both cases where TP53 IHC-positive (++ or +++) urothelium samples were found, these were located in contiguous areas as shown in Figs. 3 and 4. DNA sequencing of samples containing TP53 IHC-positive cells showed the same *TP53* mutation in three tumors

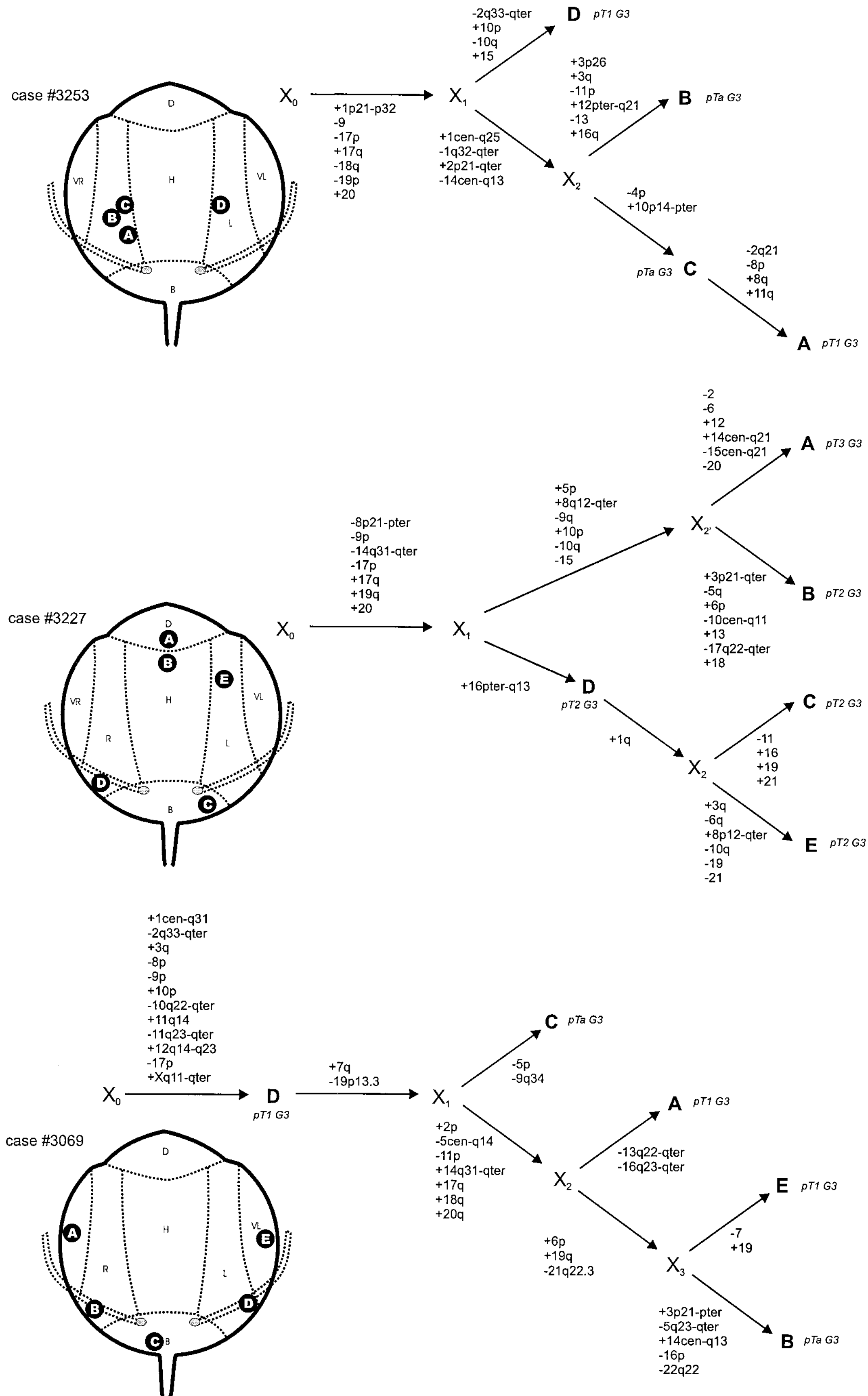

Fig. 1. Spatial and cytogenetic relationship of the tumors in six cases of multifocal bladder cancer. The localization of the individual tumors is indicated by *capital letters* inside of the bladder ideogram (*D,* bladder roof; *H,* back wall; *B,* bottom; *R,* right wall; *L,* left wall; *VR,* right front wall; *VL,* left front wall). The histological diagnosis is given in the cytogenetic pedigree of the respective case. Starting from a hypothetical precursor cell population (X_0), additional hypothetical clones (X_1–X_n) were identified based upon the highest number of identical chromosomal aberrations in addition to the precursor clone.

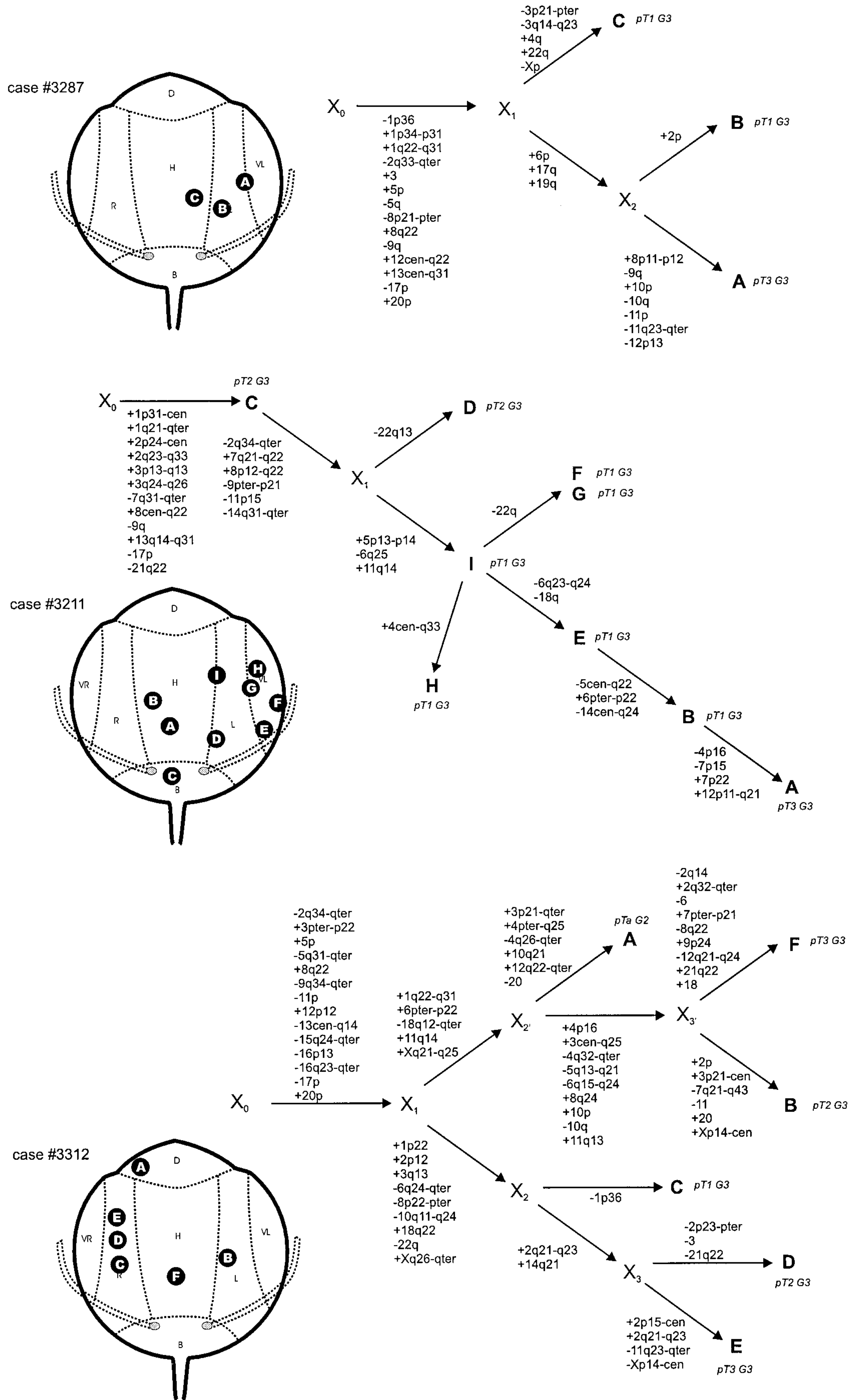

Fig. 1. *Continued.*

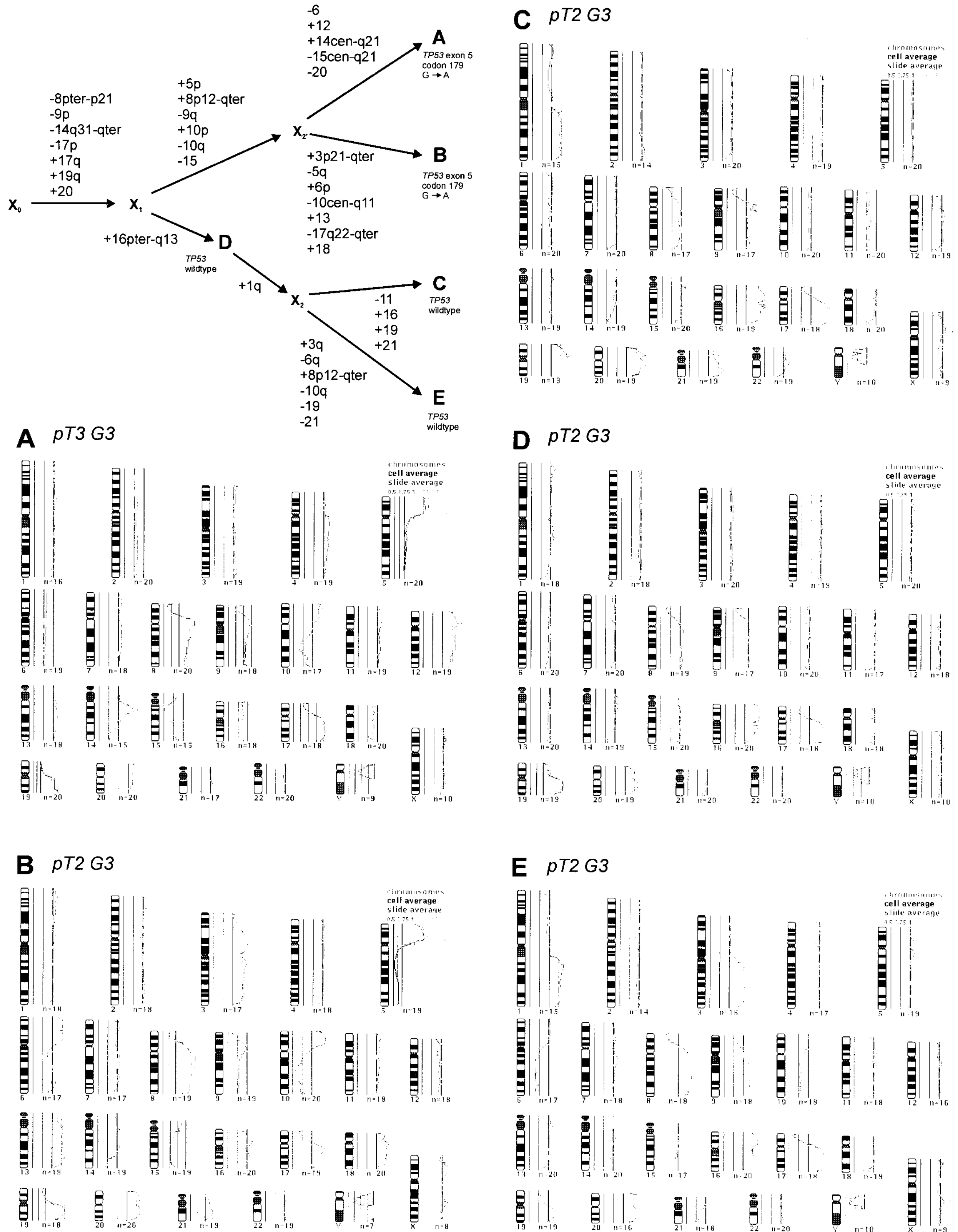

Fig. 2. CGH profiles of the tumors of case 3227 and the resulting cytogenetic pedigree demonstrating an accumulation of chromosomal aberrations during multiple tumor development. The central line of each CGH ratio profile indicates the fluorescence ratio of balanced DNA sequence copy number state (1.0) between tumor and reference DNA. *Lines* to the *left,* the 0.75 and 0.5 thresholds for losses; *lines* to the *right,* 1.25, 1.5, 1.75, and others, thresholds for gains. The ratio profiles show the mean green:red ratio (*middle line*) and the 95% confidence limits (*flanking lines*). Chromosome numbers are indicated. Tumors A and B additionally carried the same TP53 missense mutation in exon 5 codon 179, resulting in the change of GAG (glutamic acid) to AAG (lysine). A comparison with Fig. 1 demonstrates the close spatial relationship of the mutated tumors.

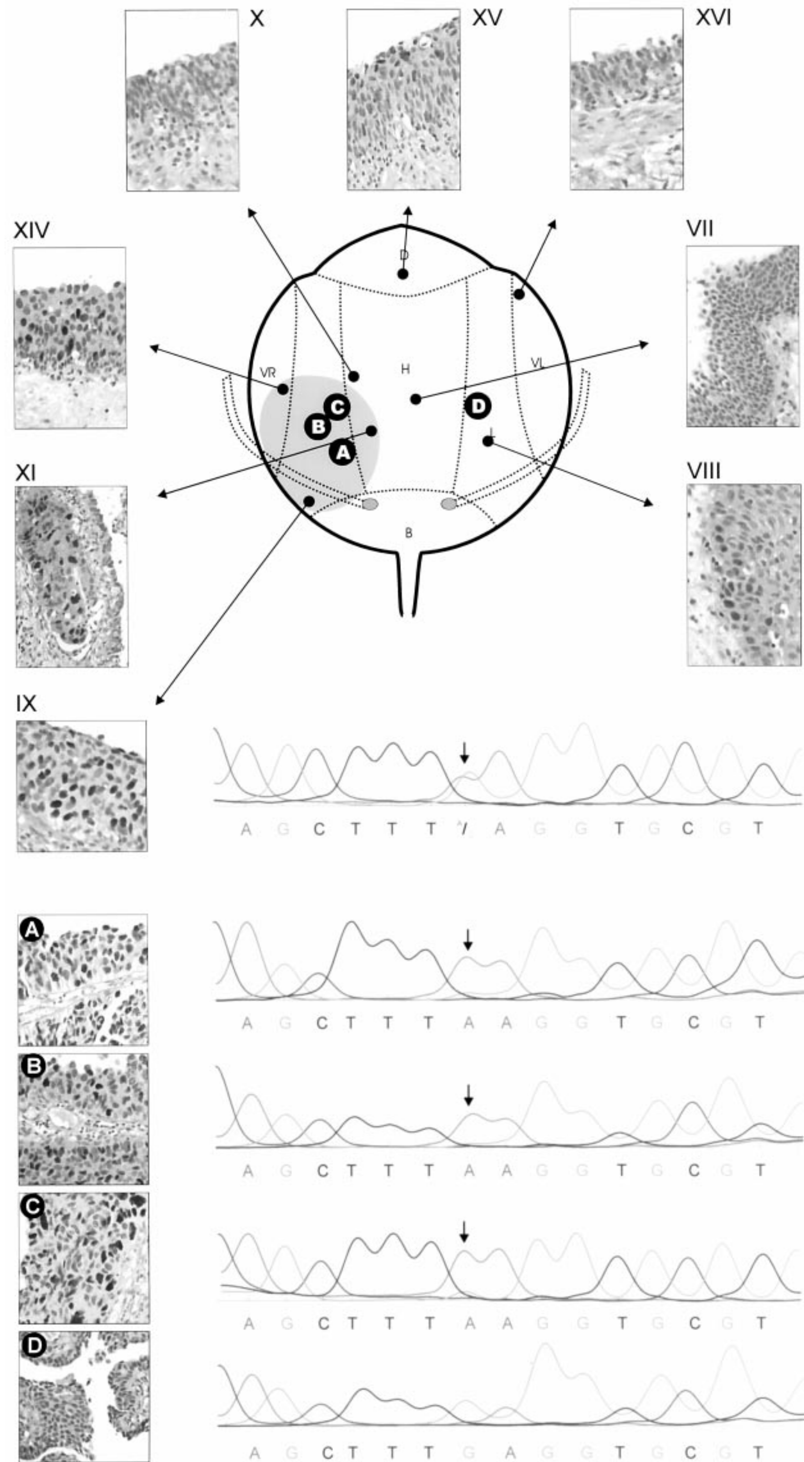

Fig. 3. TP53 sequence analysis and immunohistochemistry of case 3253. *Black circles,* the sites from which tissue samples were taken. The *gray area* inside the bladder ideogram indicates a contiguous region in which at least moderate TP53 immunostaining was detected. Sequence analysis results are displayed to the *right* of the corresponding IHC image. Sequencing revealed the same mutation in exon 8 codon 269 [resulting in the change of GAG (glutamic acid) to AAG (lysine); *arrows*] in tumors A, B, and C, whereas tumor D showed the wild-type sequence. Mucosa sample IX revealed the mutation as well as retention of the wild-type sequence, indicating a mixed population of both *TP53* mutated tumor cells and normal urothelium.

and in an adjacent mucosa sample of one of these cases. Together with the CGH findings indicating a common progenitor for the *TP53* mutant tumors, these results give strong evidence for lateral migration of neoplastic cells throughout the urothelium.

Other studies had suggested intraluminal seeding of cells shed from a primary tumor as a probable mechanism for multiple clonal tumors. Shedding of tumor cells is a frequent phenomenon and can be used for the analysis of tumor cells in urine samples (34, 35). However, intraluminal seeding is likely to require complex regulation mechanisms; cell adhesion must be lost to allow cells to shed from the primary tumor but must be retained for the process of attachment and implantation into the mucosa at a different site (36). For subsequent invasion of the urothelium, cell adhesion has to be disabled again to allow cell migration. The spread of a neoplastic clone throughout the epithelium by mucosal migration may be less complex. Although it requires loss of cell adhesion in the onset of migration mechanisms as well, there is no need for switching between cell-cell adhesion and dispersion. In addition, cells are not forced to leave the epithelial environment and to survive in the urine. However, probably most important, the spread

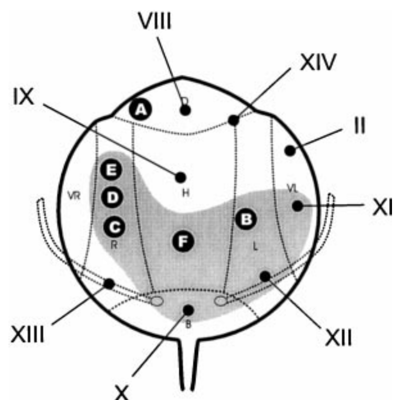

Fig. 4. TP53 immunohistochemical results of case 3312. Tumors B, C, D, E, and F (*black circles* with *capital letters*) and mucosa samples X, XI, and XII, which showed positive immunostaining, are located inside a contiguous area depicted in *gray*.

of a neoplastic clone by intraepithelial migration can be expected to lead to the formation of a single circumscribed area of transformed cells, whereas intraluminal seeding would cause a number of randomly distributed tumors. Consequently, the observation that *TP53* mutant cells were restricted to a particular area adjacent to the tumors gives strong evidence for lateral migration as the underlying mechanism for multifocal cancer development.

Taking together the cytogenetic and immunohistochemical data, it can be hypothesized that the decision whether a neoplastic cell population becomes multifocal might depend on the order in which particular genetic defects are acquired. In non-multifocal cancer, a growth advantage with tumor formation may be the initial step, followed by genetic instability, invasion capability by lysis of the lamina propria, migration into the muscularis mucosae and blood vessels attributable to loss of cell adhesion, and finally, metastatic settlement. In contrast, multiple tumors might be characterized by early genetic instability and loss of cell adhesion, leading to the migration of neoplastic cells through wide areas of the urothelium. It can be expected that this process is driven by specific genetic changes, *e.g.,* loss of 17p that might inactivate genes preventing a lateral spread of cells throughout the urothelium or maintain genetic stability. The close spatial relationship of tumors revealing identical genetic features, *e.g., TP53* mutations and patterns of chromosomal aberrations, as well as the detection of tumor cells within continuous areas of the urothelium reflect the migration of tumor cells of clonal origin throughout the bladder epithelium.

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