Microsatellite instability in multifocal urothelial carcinoma and effect on BAX and AXIN2

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Objectives: *Urothelial carcinomas have a synchronous or metachronous multifocal pattern of occurrence, questioning their clonal origin. Genetic alterations such as microsatellite instability (MSI) affect various tumors including urothelial cancers. These alterations can affect repeat sequences and cause mutations in coding regions of genes involved in transformation, tumor suppression and apoptosis. Recently, the eight-guanine (G_8) and the seven-guanine (G_7) repeat sequences of the BAX and AXIN2 genes respectively, were shown altered in different cancers. Since BAX is involved in apoptosis while the AXIN2 is involved in _-catenin metabolism, a protein involved in cell adhesion and DNA transcription, and due to the multifocal nature of urothelial cancer, we investigated these two genes for alterations in repeat sequences in patients with this cancer.*

Patients and methods: The eight microsatellites BAT25, BAT26, D2S123, D3S1029, D5S346, D17S588, D17S261, MYCL1 were used to screen 25 tumors from seven patients with eight upper and 17 lower urinary tract carcinomas and compare them to DNA from normal tissue. Regions spanning the G_8 and G_7 repeat sequences of BAX and AXIN2 were sequenced for mutations including expansion and deletion abnormalities.

Results: *Six microsatellites were seen altered in one patient with kidney and bladder cancer affecting both tissues when compared to normal DNA albeit not similarly except for MYCL1. There was no change in the BAX G_8 or AXIN2 G_7 microsatellites. There was no MSI seen in any of the remaining six patients.*

Conclusion: *MSI occurs in urothelial cancer, but was not seen to affect the BAX G_8 or AXIN2 G_7 repeats in this study. However, to determine if MSI affects these genes in these tumors will require a larger study. Moreover, our results suggest that these tumors may have a monoclonal origin with further genetic changes resulting in oligoclonality, or could suggest a similar initiating event leading to a similar initial genetic alteration at different sites with subsequent varying events due to a genetically unstable malignant phenotype.*

Key Words: urothelial cancer, BAX, AXIN2, microsatellite instability, DNA sequencing

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Introduction

Urothelial carcinoma (transitional cell carcinoma-TCC) of the upper and lower urinary tract exhibits two important characteristics: multifocality where the tumors occur in multiple distinct locations, and a high rate of recurrence.[1] This has been attributed by some to intraluminal seeding or to intraepithelial migration of cells that arise from one original clone.[2,3] Others have proposed that these multiple tumors originate

independently and are the result of independent transformation of different epithelial cells leading to independent clones.[4]

Genetic or microsatellite instability (MSI) due to defects in the DNA mismatch repair (MMR) system is now considered to be an important mechanism in carcinogenesis and has been found in primary cancers.[5] MSI manifests as expansions and deletions of simple repeated DNA sequences, leading to replication errors as seen in the hereditary nonpolyposis colorectal cancer syndrome (HNPCC), which affects the gastrointestinal and genitourinary systems.[6,7] This also results in errors in repeat sequences within coding regions of genes coding for proteins that function in apoptosis and differentiation or as cell receptors, including BAX[8,9] and AXIN2.[10] BAX which is involved in p53 activity and apoptosis, can be subject to mutations affecting the eight-guanine (G_8) microsatellite within exon three leading to alterations in codons 38-41, thus inhibiting its function.[8] AXIN2, on the other hand, along with the adenomatous polyposis coli (APC) protein and glycogen synthase kinase 3B are parts of a unit that regulates intracellular β-catenin levels,[11] which is a protein involved in cell adhesion and regulation of gene transcription. Therefore mutations in AXIN2 may alter β-catenin levels and function. The AXIN2 gene has a seven-guanine (G_7) (nucleotides 2077-2083) repeat tract in its coding region and this has been seen altered in hepatocellular carcinoma and other cancers.[12]

MSI has been seen in urothelial cancers but not in all studies. Gonzalez-Zulueta et al[13] identified MSI in 3% of all stage Ta-T1 TCC tumors studied affecting $(GT)_n$ tracts on chromosome 9 and the $(CAG)_n$ tract of the androgen receptor on the X-chromosome. In a study by Mao et al,[14] MSI was identified in 28.1% of TCC affecting only one locus, and was affiliated with TCC mainly occurring in association with HNPCC. Linnenbach et al identified MSI in only two of 40 (5%) cases of TCC of the bladder, ureter and renal pelvis, therefore describing this as a very rare event in TCC.[15] Bonnal et al[16] also found MSI to be very uncommon in urothelial cancer of the bladder. Other studies have in contrast identified a greater occurrence of this phenomenon. Uchida et al,[17] found MSI to be rare in superficial tumors, but common in invasive ones and concluded that MSI occurs and is importantly involved in tumorigenesis in TCC. Sourvinos et al[18] reported MSI in 93% of their patients affecting at least one locus, and to a lesser extent at p16, RB1 and p53 loci. Linn et al[19] detected MSI in 93% of TCC tumors and 87% of urine samples from TCC patients. Mourah et al[20] found that the MSI analysis in TCC patients had a sensitivity of 83% and specificity of 100%. Takahashi et al[21] using 21 microsatellite markers also identified MSI in urothelial carcinoma of the upper and lower urinary tract. The MSI patterns were not identical among the tumors from the same patients supporting the concept that although these tumors result from a single progenitor cell, kidney pelvis tumors are genetically more unstable than bladder neoplasms leading to spread by field cancerization.

To test these hypotheses on the patterns of spread of urothelial carcinoma of the upper urinary tract and bladder in patients who have tumors in both locations, and to investigate the role of BAX G_8 and the AXIN2 G_7 micosatellite repeats, where the latter has not been yet studied in this disease, we pursued a retrospective study of patients who had developed urothelial carcinoma of the upper and lower urinary tract. We tested for genetic alterations presenting as expansion and deletions in microsatellites including the BAX and AXIN2 repeat sequences. This allowed comparison of the extent and similarities of changes among upper and lower urinary tract tumors within the same individuals as a way to identify if the tumors derived from the same or different clones of cancer cells, and whether the two tumor suppressor genes are affected in this process. Moreover, alterations to BAX would identify a role for this gene in loss of apoptosis in urothelial cancer, a crucial step in carcinogenesis. AXIN2 mutation, on the other hand would support formation of a malignant phenotype and its ability for multifocal spread due to diminished cell adhesion capabilities.

Patients and methods

The study included seven patients who have undergone a nephroureterectomy for the treatment of urothelial carcinoma of the renal pelvis or ureter since 1997, and who subsequently developed urothelial carcinoma in the bladder, requiring tumor resection. The study was conducted following ethical approval by our Hospital's Research Review Committee, and all patients included in the study signed an informed consent.

All experimental procedures were performed using DNA from peripheral blood leukocytes, freshly acquired tumor tissue and by using tumor tissue from the paraffin blocks that are kept by the Department of Pathology at Dalhousie University with the patients' permission. Twenty five tumors of urothelial carcinoma were collected from patients with tumors

in different locations and consisted of eight tumors from the renal pelvis or ureter and 17 from the urinary bladder. Specimens obtained fresh were frozen at -70°C awaiting frozen section histologic confirmation and molecular studies. Tumor samples from the paraffin blocks were selected after reviewing the histology slides. Tumor sections were chosen based on malignant content. The leukocytes were obtained from a 10 mL blood specimen required from each tissue donor at the time of recruitment. Comparison of the sequences between the leukocytes and the tumor tissue was expected to confirm alterations in the DNA where the leukocytes served as a baseline.

Laboratory methods

DNA extraction

Peripheral blood leukocyte DNA was extracted by salt precipitation in a standard method.[22] DNA extraction from tissue samples obtained during surgery for urothelial cancer was performed as previously described.[23] Tissue embedded in paraffin blocks was extracted using Hemo-De clearing agent (Fisher Brand, Napean, ON). Briefly, samples were placed in a Hemo-De clearing agent to dissolve the paraffin and the tissue was separated and washed repeatedly with ethanol. The tissue was then incubated with 1% SDS and Proteinase-K for digestion. DNA was extracted using the IsoQuick Nucleic Acid Extraction Kit as described by the manufacturer (ORCA Research Inc., Bothell, WA). DNA was precipitated with isopropanol, washed with ethanol and redissolved in buffer for further use.

Polymerase chain reaction (PCR) amplification and microsatellites analysis

Eight microsatellite markers were used to detect microsatellite instability on various chromosomes: BAT25, BAT26, D2S123, D3S1029, D5S346, D17S588, D17S261, MYCL1.[24,25] These markers were analyzed by polymerase chain reaction (PCR) amplification followed by electrophoretic separation.[24-26] The PCR for each microsatellite were performed in 30 uL volumes using primer sequences obtained from the Genome Database, 5U of Taq polymerase and ^{32}P dCTP as a radioactive label. For analysis of BAT25 and BAT26, the procedure consisted of an initial denaturation at 95°C for 1.5 min, followed by 30 cycles of denaturation at 93°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. The procedure for the remaining microsatellites consisted of an initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturing at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes. A final extension step at 72°C for 7 minutes completed the DNA amplification process. Each radiolabelled amplicon was then heated at 65°C for 10 minutes to denature the DNA and electrophoresed on an 8M urea gel. The gel was then transferred onto filter paper, dried for 30 minutes at 80°C and autoradiographed at -70°C for 18 hours.

Polymerase chain reaction amplification and sequencing of the BAX G_8 and AXIN2 G_7 microsatellites

For detection of microsatellite instability of the BAX G_8 repeat sequence, analysis was performed as previously described by us with a minor modification.[27] Briefly, using forward (5'-ATCCAGGATCGAGCAGGGCG-3') and reverse (5'-GACAGTAACATGG-AGCTGCA) primers, a 140 bp fragment was amplified using a thermal cycler with an initial cycle at 95°C for 1.5 minutes, followed by 30 cycles of denaturing at 93°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. A final primer extension at 72°C completed the reaction. The fragment was amplified in a reaction volume of 30 uL using 30 pmols of each primer, and 1U Taq DNA polymerase, 50 ng of genomic DNA and 1.25 mM of each dNTP. The PCR product was then purified using a GFX column. For all patients negative by the above eight microsatellite analysis, the G_8 repeat of each purified PCR product was analyzed using Sanger's dideoxy chain termination method and sequencing the guanine lane only.[23,27] A Thermo Sequencing Radiolabelled Terminator Cycle Sequencing Kit (USB, Amersham Life Sciences, Ohio) was used in the analysis. The forward PCR primer was used as the sequencing primer. Each reaction sample was run on a 6% polyacrylamide gel and autoradiographed. AXIN2 G_7 repeat was similarly amplified and sequenced.[12] The following forward (5'-CTG-GGTGAACAGGTGGGCACG-3') and reverse (5'-CCGCTCGTCTCCAGGCGAACG-3') primers were used. The PCR protocol included an initial denaturation step at 95°C for 1.5 minutes followed by 30 cycles of denaturing 93°C for 30 seconds, annealing at 64°C for 1 minute and extension at 72°C for 1 minute. In the case of one patient who was positive with some of the eight microsatellite markers, a full sequencing reaction was performed looking at all four nucleotides.

Results

Patients consisted of six males and one female and they ranged in age from 64 to 72 years. Tumor grades

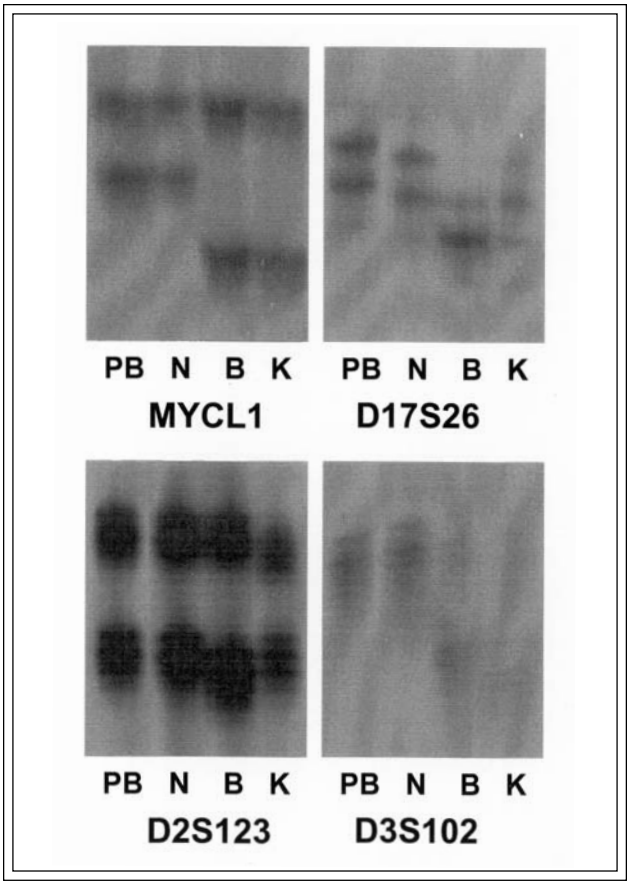

Figure 1. Microsatellite analysis showing concordant and discordant genetic alterations in a multifocal tumor. The radiographs show comparison of DNA from peripheral blood leukocytes (PBL), normal mucosa (NM), bladder tumor (BT) and kidney tumor (KT). Identical microsatellite shift patterns are seen with MYCL1, while discordant shifts are seen in D17S261, D2S123 and D3S1029.

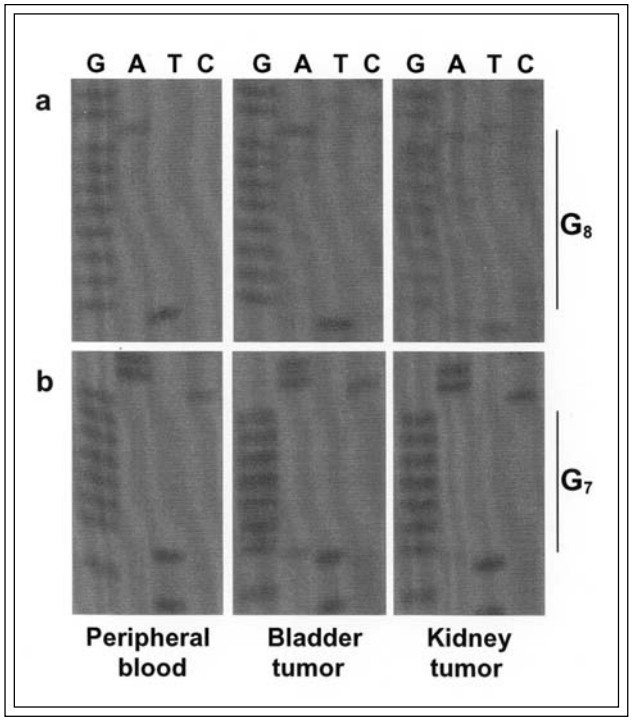

Figure 2. DNA sequence analysis of the BAX (a) and AXIN2 (b) genes by Sanger's dideoxy chain termination method of peripheral blood leukocytes (control), and bladder and kidney tumors. The G8 and G7 microsatellite repeats of BAX and AXIN2 respectively are unaffected and remain stable.

ranged from I to III. Microsatellite analysis confirmed alterations in six microsatellites in only one of the seven patients studied. This instability affected the kidney pelvis and bladder tumors but not always similarly where detected. The microsatellites affected were MYCL1, D17S261, D2S123, D3S1029 Figure 1, and BAT26 and D17S588 (not shown), and MYCL1 changes were similar in both tissues. Microsatellite analysis of the BAX G_8 and AXIN2 G_7 repeat tracts by sequence analysis demonstrated the normal sequence and position of the eight-guanine residues in the BAX gene in the peripheral blood leucocytes, bladder tumors or kidney pelvis tumors in all seven patients including the one with the microsatellite instability described above and shown in Figure 2. The AXIN2 G_7 tract was equally stable in all patients. In summary, microsatellite analysis showed instability in one of seven patients with urothelial cancer of the bladder and kidney pelvis, but not resulting in any instability in the BAX G_8 or the AXIN2 G_7 microsatellites. The instability was not similar in some microsatellites between the kidney and bladder supporting the hypothesis that these tumors may have a monoclonal origin with further genetic alteration resulting in oligoclonality as suggested by others, or a multifocal origin resulting from a similar genetic change (MYCL1) with further diversification due to the instability of this malignant phenotype.

Discussion

Urothelial cancer exhibits two very important characteristics, multifocality and a high rate of recurrence.[1] The risk of recurrence in the bladder, even with radical surgery of the upper urinary tract, is between 15% and 45%. Two hypotheses were proposed to explain the multifocal nature and high rate of recurrence, the monoclonal origin hypothesis which attributes the multifocality to intraluminal

seeding or intraepithelial migration of one clone of transformed cells giving rise to daughter cells, all exhibiting the same genetic change that initially provided the growth advantage to the parent cell. This view is supported by Sidransky et al who identified similar genetic alterations amongst multifocal bladder tumors existing within one patient and leading to topologically distinct but genetically related tumors.[2,3,28] The field defect hypothesis on the other hand suggests that a carcinogenic insult results in the independent transformation of many epithelial cells leading to independent clones which form multiple new tumors in various regions of the urinary tract. That is, all tumors originate independently,4 suggesting that chemical carcinogens initiate the proliferation of many clones of cells and that subsequent promotion of growth by carcinogens or inflammatory factors may lead to the formation of multifocal tumors, either synchronous or metachronous.[17]

To study the development and progression of these tumors, some studies have investigated the stability and homogeneity of microsatellite repeats in TCC and among the multiple tumors developing within the same patient. It was considered that similar MSI changes in these tumors would confirm the monoclonal hypothesis. On the other hand, tumors arising independently would show different MSI patterns. In effect, different studies have produced conflicting observations. Earlier studies using molecular techniques mostly favor the monoclonal hypothesis. Sidransky et al[2] examined X-chromosome inactivation and chromosome 9q, 17p and 18q allelic losses, and found that all tumors had inactivation of the same chromosome in addition to the early loss of the same allele on chromosome 9. While loss of chromosomes 17p and 18q was a late event in tumor progression, loss of 9q preceded the spread of tumor cells elsewhere in the bladder. Simon et al[29] reported that multifocal tumors arising from the same patient showed the same identical chromosomal aberrations. Additionally, identical genetic alterations in contiguous but normal mucosa samples pointed at intraepithelial migration. Tsao et al[30] found that tumors maintained the allelic losses at specific chromosomes with little or no increase with time. Takahashi et al[31] using microsatellite markers on chromosomes 2q, 4p, 4q, 8p, 9q, 11p, and 17p in multifocal tumors affecting the bladder, renal pelvis and ureter, concluded from the patterns of loss of heterozygosity, MSI patterns and subchromosomal deletions that at least 80% of tumors originate from a single progenitor cell, while multifocal tumors in a small subset of patients might develop from distinct origins due to field cancerization.

Some argue that the reason for favoring the monoclonal origin hypothesis in the earlier studies, was probably due to the fact that most studies were limited to the study of advanced and poorly differentiated tumors of the bladder. Some studies now provide evidence for oligoclonality of urothelial tumors. Paiss et al[32] examined tumors for X-chromosomal inactivation and identified a polyclonal origin in 36%. Hartmann et al[33] identified considerable genetic heterogeneity suggesting initial oligoclonality. Takahashi et al[21] identified similar MSI in different locations in some patients, indicating that upper and subsequent lower urothelial tumors result from a single progenitor cell by seeding or intraepithelial spread causing multifocal development. However, they also observed some discordance in MSI and indicated that upper urinary tract neoplasms are genetically more unstable than bladder neoplasms thus supporting the field cancerization mechanism. Hafner et al[34] found deletions of chromosome 9 in 73% of tumors and on 17p13 in 18%, suggesting a monoclonal pattern, with the same p53 mutation in all affected tumors. Some patients however, showed different upper and lower tract patterns suggesting an oligoclonal origin and that in some bladder cancer developed before the upper urinary tract cancer. They concluded that most multifocal urothelial tumors are monoclonal, however some are oligoclonal, which supports field cancerization. Moreover, urothelial cancer seemed to be the result of monoclonal tumor spread by intraluminal seeding leading to the development of distinct tumors.

BAX is a member of the BCL2 family of proteins and is believed to be involved in regulating apoptosis by forming a channel in the mitochondrial membrane.[8] Loss of function by mutation leads to loss of apoptosis and carcinogenesis. A mechanism for selective vulnerability of BAX for mutations is provided by the presence of a G_8 mononucleotide repeat tract in the third exon of its coding region. It is known that insertions or deletions of the G_8 tract of the BAX gene may be seen in association with a defective mismatch repair system and MSI. We hypothesized that a subset of urothelial cancers may have a mutated G_8 tract of the BAX gene, but the G_8 tract of BAX showed stability in all our patients indicating that the microsatellite in BAX was not affected. This may be supported by immunohistochemical evidence that most of urothelial tumors show immunoreactivity for BAX.[35,36]

Similar to the BAX G_8 tract, the G_7 tract of the AXIN2 gene has been associated with MSI in some cancers. The role of AXIN2 in regulating β-catenin levels, a protein involved in gene expression, differentiation and cell adhesion, would support the hypothesis that loss of AXIN2 may lead to urothelial cancer formation and multifocal spread due to loss of cell adhesion capability among these tumors. However, in this study, the G_7 microsatellite of the AXIN2 gene was not found to be unstable in any of the patients. Other studies may support these findings which also show that the Wnt signalling pathway, and not only AXIN2, are rarely affected in urothelial carcinoma. For example, the AXIN gene was not significantly affected in pediatric renal tumors.[37] Additionally, only the APC and not the β-catenin gene was barely affected in bladder tumors.[38] This supports the suggestion that AXIN2 may not play a role in the majority of urothelial cancers and may validate our conclusion that the AXIN2 G_7 repeat is stable in this disease.

Review of previous studies shows conflicting data on the role of MSI in urothelial carcinomas and the monoclonal or oligoclonal nature of these tumors. Our study of peripheral blood, normal tissue, and tumorous renal pelvis and bladder in seven patients, showed that only one of those had MSI (14%). This instability was seen using the microsatellites BAT26, D2S123, D3S1029, D17S588, D17S261, MYCL1. Therefore, this confirms the presence of MSI in urothelial carcinoma albeit at a lesser rate than seen by others. In the single affected patient, only one microsatellite showed the identical change in the renal pelvic tissue and the bladder, namely the MYCL1 locus. The other five unstable loci among the remaining seven tested showed differing MSI patterns between the tumors from both locations. The identical MYCL1 microsatellite pattern can support previous studies showing that most multifocal urothelial tumors can originate from a single or identical progenitor cells independent at the initial site of the disease. However, the change in the remaining microsatellites identifies further diversification within these tumors as they spread. On the other hand, it may also suggest that a similar initiating event could have led to a similar genetic alteration at different sites, with acquisition of different subsequent genetic changes, due to the genetic instability resulting from this form of malignant phenotype. However, the two microsatellites in BAX and AXIN2 were not affected by these changes in any of the patients including the patient with MSI indicating the lack of involvement of these sequences in urothelial cancer. □

References

1. Bender CM, Jones PA. Carcinoma of the Bladder: Innovations in Management. Petrovich Z, Baert L and Brady LW (ed). Springer: Heidelberg/New York, pp37-51, 1998.
2. Sidransky D, Frost P, Von Eschenbach A, Oyasu R, Preisinger AC, Vogelstein B. Clonal origin of bladder cancer. *New Engl J Med* 1992;326:737-740.
3. Chern H, Becich MJ, Persad RA, Romkes M, Smith P, Collins C, Li YH, Branch RA. Clonal analysis of human recurrent superficial bladder cancer by immunohistochemistry of p53 and retinoblastoma proteins. *J Urol* 1996;156:1846-1849.
4. Richie JP, Shipley WU, and Yogada A. Cancer of the bladder. In: Cancer: Principles and Practise of Oncology. Devita VT, Hellman S, and Rosenberg (eds). JB Lippincott: Philadelphia, vol 1, pp1008-1022, 1989.
5. Rhyu MS. Molecular mechanisms underlying hereditary nonpolyposis colorectal carcinoma. *JNCI* 1996;88:240-249.
6. Lazar V, Grandjouan S, Bognel C et al. Accumulation of multiple mutations in tumor suppressor genes during colorectal tumorigenesis in HNPCC patients. *Hum Mol Genet* 1994;3:2257-2260.
7. Gurin CC, Federici MG, Kang L, Boyd J. Causes and consequences of microsatellite instability in endometrial carcinoma. *Cancer Res* 1999;59:462-466.
8. Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC, Perucho M. Somatic frameshift in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* 1997;275:967-969.
9. Abdel-Rahman WM, Georgiades IB, Curtis LJ, Arends MJ, Wyllie AH. Role of BAX mutations in mismatch repair-deficient colorectal carcinogenesis. *Oncogene* 1999;18:2139-2142.
10. Clevers H. Axin and hepatocellular carcinoma. *Nature Genet* 2000;24:206-208.
11. Kawahara K, Morishita T, Nakamura T, Hamada F, Toyoshima K, Akiyama T. Down-regulation of beta-catenin by the colorectal tumor suppressor APC requires association with Axin and beta-catenin. *J Biol Chem* 2000;275:8369-8374.
12. Liu W, Dong X, Mai M, Seelan RS, Taniguchi K, Krishnadath KK, Halling KC, Cunningham JM, Qian C, Christensen E, Roche DI, Thibodeau SN. Mutations in *AXIN2* cause colorectal cancer with defective mismatch repair by activating b-catenin/TCF signalling. *Nature Genet* 2000;26:146-147.
13. Gonzalez-Zulueta M, Ruppert JM, Tokino K, Tsai YC, Spruck III HC, Miyao N, Nichols PW, Hermann GG, Horn T, Steven K, Summerhayes IC, Sidransky D, Jones PA. Microsatellite instability in bladder cancer. *Cancer Res* 1993;53:5620-5623.
14. Mao L, Lee DJ, Tockman MS, Erozan YS, Askin F, Sidransky D. Microsatellite alterations as clonal markers for the detection of human cancer. *Proc Natl Acad Sci USA* 1994;91:9871-9875.
15. Linnenbach AJ, Robbing SL, Seng BA, Tomaszewski JE, Pressler LB, Malkowicz SB. Urothelial carcinogenesis. *Nature* 1994;367:419-420.
16. Bonnal C, Ravery V, Toublanc M, Bertrand G, Boccon-Gibod L, Henin D, Grandchamp B. Absence of microsatellite instability in transitional cell carcinoma of the bladder. *Urology* 2000;55:287-291.
17. Uchida T, Wang C, Wada C, Iwamura M, Egawa S, Koshiba K. Microsatellite instability in transitional cell carcinoma of the urinary tract and its relationship to clinicopathological variables and smoking. *Int J Cancer* 1996;69:142-145.
18. Souvrinos G, Kazanis J, Delakas D, Cranidis A, Spandidos DA. Genetic detection of bladder cancer by microsatellite analysis of p16, RB1 and p53 tumor suppressor genes. *J Urol* 2001;165:249-252.

19. Linn JF, Lango M, Halachmi S, Schoenberg MP, Sidransky D. Microsatellite analysis and telomerase activity in archived tissue and urine samples of bladder cancer patients. *Int J Cancer* 1997;74:625-629.
20. Mourah S, Cussenot O, Vimont V, Desgrandchamps F, Teillac P, Cochant-Proillet B, Le Duc A, Fiet H, Soliman H. Assessment of microsatellite instability in urine in the detection of transitional-cell carcinoma of the bladder. *Int J Cancer* 1998;79:629-633.
21. Takahashi T, Kakehi Y, Mitsumori K, Akao T, Terachi T, Kato T, Ogawa O, Habuchi T. Distinct microsatellite alterations in upper urinary tract tumors and subsequent bladder tumors. *J Urol* 2001;165:672-677.
22. Miller SA, Dykes DD, Polesky HF. A simple salting out method for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16:1215.
23. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, 1989.
24. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S. A national cancer institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res* 1998;58:5248-5257.
25. Jass JR, Cottier DS, Jeevaratnam P, Pokos V, Holdaway KM, Bowden ML, Van de Water NS, Browett PJ. Diagnostic use of microsatellite instability in hereditary non-polyposis colorectal cancer. *Lancet* 1995;346:1200-1201.
26. Orita M, Iwahana H, Kanazawa H, hayashi K, Sekiya T. Detection of polymorphisms of human DNA by gel electrophoresis as single strand conformation polymorphisms. *Proc Natl Acad Sci USA* 1989;86:2766-2770.
27. Sanger F, Nicklin S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 1977;74:5463-5467.
28. Sidransky D, Messing E. Molecular genetics and biochemical mechanisms in bladder cancer. *Urol Clin North Am* 1992;19:629-639.
29. Simon R, Eltze E, Schafer KL, Burger H, Semjonow A, Hertle L, Dockhorn-Dworniczak B, Terpe HJ, Bocker W. Cytogenetic analysis of multifocal bladder cancer supports a monoclonal origin and intraepithelial spread of tumor cells. *Cancer Res* 2001;61:355-362.
30. Tsao JL, Yatabe Y, Markl ID, Hajyan K, Jones PA, Shibata D. Bladder cancer genotype stability during clinical progression. *Genes Chromosomes Cancer* 2000;29:26-32.
31. Takahashi T, Habuchi T, Kakehi Y, Mitsumori K, Akao T, Terachi T, Yoshida O. Clonal and chronological genetic analysis of multifocal cancers of the bladder and upper urinary tract. *Cancer Res* 1998;58:5835-5841.
32. Paiss T, Wohr G, Hautmann RE, Mattfeldt T, Muller M, Haeussler J, Vogel W. Some tumors of the bladder are polyclonal in origin. *J Urol* 2002;167:718-723.
33. Hartmann A, Rosner U, Schlake G, Dietmaier W, Zaak D, Hofstaedter F, Knuechel R. Clonality and genetic divergence in multifocal low-grade superficial urothelial carcinoma as determined by chromosome 9 and p53 deletion analysis. *Lab Invest* 2000;80:709-718.
34. Hafner C, Knuechel R, Zanardo L, Dietmaier W, Blaszyk H, Cheville J, Hofstaedter F, Hartmann A. Evidence for oligoclonality and tumor spread by intraluminal seeding in multifocal urothelial carcinomas of the upper and lower urinary tract. *Oncogene* 2001;20:4910-4915.
35. Korkolopoulou P, Lazaris ACh, Konstantinidou AE, Kavantzas N, Patsouris E, Christodoulou P, Thomas-Tsagli E, Davaris P. Differential expression of bcl-2 family proteins in bladder carcinomas. Relationship with apoptotic rate and survival. *Eur Uro1* 2002;4:274-283.
36. Wolf HK, Stober C, Hohenfellner R, Leissner J. Prognostic value of p53, p21/W AF1, Bcl-2, Bax, Bak and Ki-67 immunoreactivity in pT1 G3 urothelial bladder carcinomas. *Tumour Bio1* 2001;22:328-336 .
37. Maio J, Kusafuka T, Udatsu Y, Okada. Axin, the main component of the Wnt signaling pathway, is not mutated in kidney tumors in children. *Int J Mol Med* 2002;9:377-379.
38. Stoehr R, Krieg RC, Knuechel R, Hofstaedter F, Pilarsky C, Zaak D, Schmitt R, Hartmann A. No evidence for involvement of beta-catenin and APC in urothelial carcinomas. *Int J Oncol* 2002;20:905-911.