

Alteration of the *LRP1B* Gene Region Is Associated with High Grade of Urothelial Cancer

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SUMMARY: We have delineated regions of interest at chromosome 2q21.2, 2q36.3, and 2q37.1 by deletion mapping of 114 urothelial cancers (UC). Altogether, 17%, 18%, and 63% of the G1, G2, and G3 tumors displayed loss of heterozygosity at chromosome 2q, respectively. The region at 2q21.2 was narrowed down to the *LRP1B* gene (NT_005129.6). Hemi- and homozygous deletion at the *LRP1B* gene region was seen in 31 of 114 UCs. Only 8% of the UCs with G1 and none with G2 tumors showed loss of heterozygosity at the *LRP1B* gene, whereas 49% of the G3 UCs had allelic loss at this region. RT-PCR analysis of the *LRP1B* gene showed the lack of expression of several exons in 2 of 9 cases analyzed. Our analysis suggests that the *LRP1B* gene is a candidate tumor suppressor gene in UCs. (*Lab Invest* 2002, 82:639–643).

Cancer of the urinary bladder is one of the most common tumors in the Western world. The majority of urothelial cancers (UC) are diagnosed as noninvasive tumors (Ta), whereas 20% to 25% of the cases show an invasive growth (T1–4) at the time of first presentation. From the clinical point of view, the question arises whether these two major groups of tumors are distinct entities or correspond to different stages of progression of a single tumor entity. Pioneering cytogenetic analyses before the chromosome banding era have suggested that the number of gross karyotype alterations predicts the clinical course of UCs, for example, recurrency and progression (Falor and Ward, 1978; Lamb, 1967). Later, several studies showed that allelic changes at specific chromosomal regions and alterations of tumor suppressor genes, such as *PTEN*, *RB*, and *TP53*, correlate with stage and grade of bladder cancers (for review see Knowles, 1999). Comparative genomic hybridization (CGH) studies also suggested quantitative differences of genetic changes, including DNA losses at chromosome 2q22–33, 2q32–qter, and 2q34–qter regions, between the noninvasive and invasive bladder cancers (Richter et al, 1997, 1999; Simon et al, 1998, 2000). Loss of heterozygosity (LOH) at chromosome 2q is also associated with aggressive growth of head

and neck and non-small cell lung carcinomas (Ransom et al, 1998; Shiseki et al, 1994). Recently, Liu et al (2000) identified a putative tumor suppressor gene *LRP1B* from the chromosome 2q21.2 region that was found to be homozygously deleted in several cancer cell lines, including the bladder cancer cell line VM-CUB-2. To delineate putative tumor suppressor gene regions, we analyzed 114 UCs for 20 microsatellite loci at the chromosome 2q including those from the *LRP1B* region. We identified three distinct regions of LOH in 40% of tumors and found a correlation between LOH at chromosome 2q and tumor grade.

Results

Three Target Regions of Allelic Loss at Chromosome 2q

CGH analysis of 18 Grade 3 (G3) UCs of this series revealed a gain at chromosome 2p in 7 cases and loss of DNA at chromosome 2q in 11 cases (one example is shown in Fig. 2). Therefore, we evaluated score 2 at chromosome 2p as a duplication of one allele, but at chromosome 2q as a loss of heterozygosity (LOH) occurring in mosaic form. Allelic changes at chromosome 2p were detected in 23 (20%) of 114 UCs. The deletion mapping delineated three regions of interest at chromosome 2q (Fig. 1). Region A was narrowed down between loci D2S150 and tetra 30 encompassing loci D2S2286, D2S2239, and D2S129, which are localized to the 1,230 kb genomic region of the *LRP1B* gene (NT_005129.6). Region B is localized to an approximately 1.5-Mb genomic segment between loci D2S130 (NT_005126.5) and D2S2228 (NT_005106.6), including the *CUL3* gene. Region C is mapped between loci D2S2297 (NT_005403.6) and D2S172

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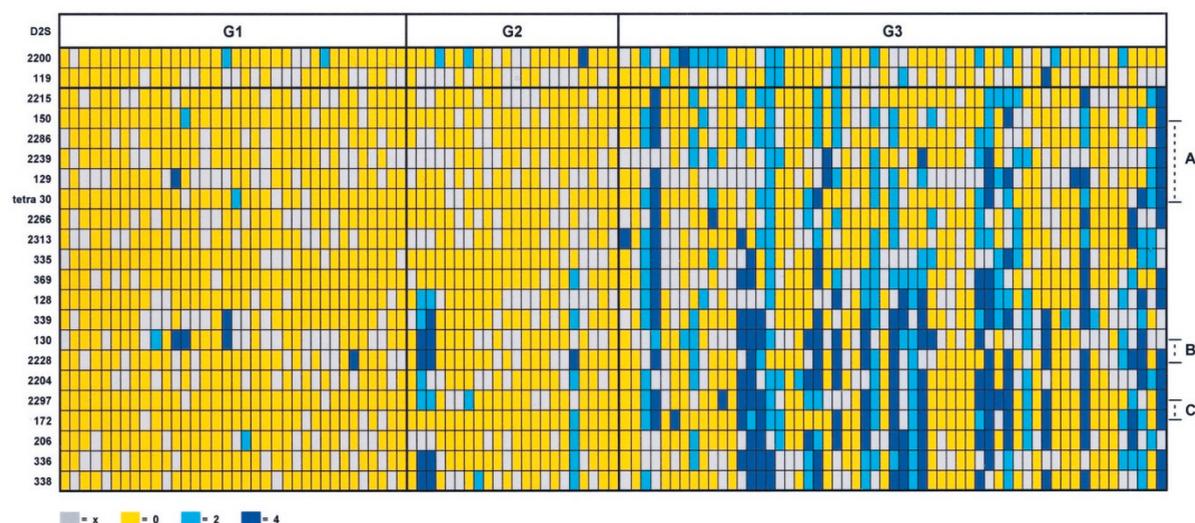


Figure 1.

Summary of microsatellite analysis of 114 bladder cancers. Microsatellite loci are listed on the left side. Retention of heterozygosity is marked with yellow, allelic imbalance with light blue, loss of heterozygosity with dark blue, and noninformative loci with gray. The three regions of interest (A to C) are shown on the right side. Only five of the G1 urothelial tumors showed an allelic changes at 2q, whereas loss of heterozygosity at these regions occurred at high frequency in G3 cancers.

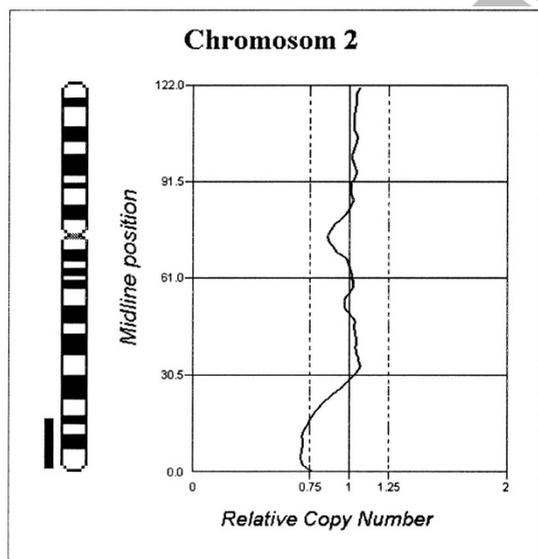


Figure 2.

Comparative genomic hybridization analysis of a urothelial carcinoma showing DNA loss at chromosome 2q33-qter.

NT_026242.3), which spans approximately a 1.8-Mb genomic segment. Altogether, LOH involving at least one of these regions was seen in 46 (40%) of the 114 bladder cancers.

LOH at 2q Is Associated with High Grade of UCs

Alterations at regions A, B, and C occurred with increasing frequency in tumors from Stage Ta through T1 to T2–4 (Table 1). The correlation between stage and LOH was similar for all regions (data not shown). Altogether, LOH at chromosome 2q occurred in 20%, 60%, and 57% of Ta, T1, and T2–4 UCs, respectively. Evaluation of the genetic alterations at region A re-

Table 1. Loss of Heterozygosity at Chromosome 2q and Stage or Grade of Urothelial Cancer

Grade	Tumor stage			Total ^a
	pTa	pT1	pT2–4	
1	6/34	–/1	–	6/35 (17)
2	3/17	1/4	–/1	4/22 (18)
3	2/3	11/15	23/39	36/57 (63)
Total ^a	11/54 (20)	12/20 (60)	23/40 (57)	46/114 (40)

^aNumbers in brackets indicate the percentage of allelic changes.

garding the grade of tumors showed a cutoff between G1–G2 versus G3 tumors. Only 3 of 35 G1 UCs and none of the 22 G2 tumors showed LOH at the *LRP1B* gene. In contrast, 28 of 57 G3 tumors showed allelic loss at this region. No LOH was found at region C and D in G1 tumors, whereas G2 and G3 UCs showed LOH in 14% to 18% and 44% to 49% of the cases, respectively. Altogether, 17% and 18% of the G1 and G2 tumors displayed LOH at chromosome 2q, compared with 63% LOH in G3 UCs (Fig. 1; Table 1).

Involvement of the *LRP1B* Gene

LOH including the *LRP1B* gene region was seen in 31 of 114 UCs, including 21 cases with partial deletions occurring exclusively at the *LRP1B* gene. The 91 exons of the *LRPB1* are distributed along a 1,230 kb genomic region between position 229 kb and 1,459 kb in the sequence segment NT_005129.6. Loci D2S2286, D2S2239, and D2S129 are mapped to positions 604 kb, 966 kb, and 1,172 kb, respectively, therefore being in the *LRPB1* genomic region. The loci tetra30 and D2S2266 are mapped to position 1,797 kb and 1,837 kb, respectively, which are outside the *LRPB1* region. Locus D2S2313 is localized approximately 400 kb distally (NT_005102.6) and locus

D2S150 is mapped about 270 kb proximally to the *LRP1B* gene region (NT_029242.1). The LOH data indicate clearly that a hemizygous deletion affects the *LRP1B* gene region in 27 cases. Allelic imbalance was detected at all informative loci along the chromosome 2q excepting of loci D2S2286, D2S2239, and tetra30 in one, one, and two UCs, respectively. These data indicate a possible homozygous deletion at these loci. The results of the RT-PCR analysis in primary UC tissues also indicate the involvement of *LRP1B* in UC. Although the *LRP1* gene showed a strong signal in all normal urothelial and tumor samples, the PCR product from *LRP1* exons 1, 8, 10, and 29 was missing in tumor 157 and exons 1, 8, and 29 in tumor 101 (Fig. 3). RT-PCR analysis showed expression of the *CUL3* gene in all normal and tumor tissues (not shown).

Discussion

We delineated three regions of LOH at chromosome 2q21.2, q36.3, and q37.1 by a detailed microsatellite analysis of 114 UCs. The region at chromosome 2q21.2 was restricted to the *LRP1B* gene. Lisitsyn et al (1995) found homozygous deletion within this region in cell lines established from distinct types of tumors including bladder cancer. Recently, these authors have detected homozygous deletion at one or more exons of the *LRP1B* gene in several lung cancer cell lines (Liu et al, 2000). Our data showed that in UCs, a hemizygous deletion is the most common genetic alteration within the *LRP1B* gene region, but in some cases a homozygous deletion may occur. The RT-PCR analysis of the 5'-end, the middle part, and the 3'-end of the coding sequences of the *LRP1B* gene indicated the lack of transcripts in some UCs. Because the large number of exons of the *LRP1B* gene we did not sequence genomic DNA for mutation analysis. The genetic data and pathologic parameters suggest that inactivation of the *LRP1B* gene is associated with high grade, invasive growing UCs.

Region B harbors the *CUL3* gene (a member of the cullin/Cdc53 protein family), and five hypothetical proteins (KIAA0694, FLJ20220, FLJ22746, FLJ12701, and KIAA1435). Overexpression of the *CUL3* in-

creases the ubiquitination of cyclin E, whereas deletion of the *CUL3* leads to an accumulation of the cyclin E protein in cells and consequently to a high proportion of cells in the S-phase (Singer et al, 1999). *CUL3* is ubiquitously expressed in normal and UC cells and likely another gene might be the target of LOH at this region. Region C harbors the *SP140* gene (nuclear body protein Sp140), the hypothetical genes *LOC2737*, *LOC92739*, *LOC116397* and *LOC82459*, as well as the hypothetical protein FLJ20701. No data are available on these genes in bladder cancer.

Previously, CGH analyses revealed chromosome 2q alterations in 3% to 9% of UCs with Stage Ta tumors (Richter et al, 1997; Simon et al, 1998; Zhao et al, 1999), and in 17% to 36% of the T1 tumors (Richter et al, 1997, 1998, 1999; Simon et al, 1998). Based on CGH data, a major genetic difference between the noninvasive Ta and invasive T1–4 tumors, as well as a high degree of genetic instability in invasive tumors, was suggested (Sauter and Mihatsch, 1998). Although we found a correlation between LOH at chromosome 2q and tumor staging, our study clearly shows that alteration of chromosome 2q is primarily associated with the grade of UCs. We found a cutoff between G1–G2 versus G3 UCs of the bladder. The higher frequency of LOH in invasive UCs (T1–4) is associated with the high proportion of G3 tumors in this group as shown in Table 1. Previously, we obtained a similar correlation between LOH and grade of bladder cancer by analyzing the chromosome 8p23.3 and chromosome 5q regions (Muscheck et al, 2000; von Knobloch et al, 2000).

The stage, eg, the invasive growth, of a malignant tumor relies on the interaction between tumor cells and their local environment, as well as on the host reaction against the tumor cells. The grading system reflects the proliferative activity of tumor cells themselves. Our allelotyping study suggests that loss of chromosome 2q, especially at the *LRP1B* gene, is primarily associated with the malignant proliferation of urothelial cells. Identifying relevant genes from the target regions, such as the gene *LRP1B*, will be helpful in molecular grading of UCs.

Materials and Methods

Tumor Samples and DNA Extraction

Fresh tumor tissues were obtained by transurethral resection or radical cystectomy at the Departments of Urology, Philipps-University Marburg and Mannheim Clinic, Ruprecht-Karls-University Heidelberg. All tumors were histologically reevaluated according to the World Health Organization Classification (Mostofi et al, 1999) by two of the authors (GK, ALB). This study comprised 54, 20, and 57 tumors of pathologic Stage Ta, T1, and T2–4, respectively. Grades 1, 2, and 3 were identified in 35, 22, and 57 cases, respectively (Table 1). A part of the tumor tissue was immediately snap-frozen in liquid nitrogen and stored at -80°C . A frozen tumor sample was placed on a plastic Petri dish, covered with 1 ml TE9 buffer, and allowed to

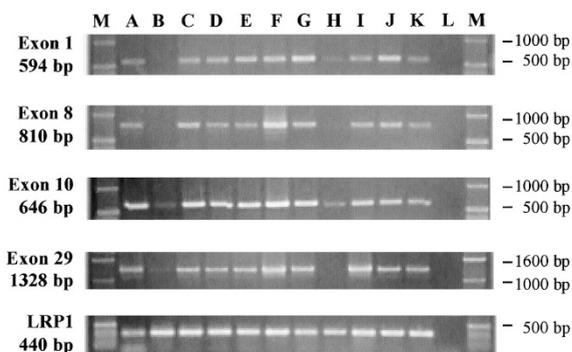


Figure 3.

RT-PCR analysis of the 5'-end, the middle part, and 3'-end of the coding sequences of the *LRP1B* gene in normal urothelium (J, K) and urothelial cancers (A to I). Note the lack of signal in tumors B and H.

thaw. The tumor cells were then carefully scraped off the transurethral resection samples and pushed out from cystectomy specimens under an inverted microscope by a pathologist (GK) experienced in this technique. By this method, contamination with normal cells was reduced to a minimum. Afterward, the tumor cells were resuspended in 2 to 5 ml TE9 buffer with 1% SDS and 0.2 mg/ml proteinase K and were incubated for 8 to 12 hours at 55° C. DNA was extracted by phenol-chloroform and dissolved in TE buffer after ethanol precipitation. Normal control DNA was extracted from blood lymphocytes by the same method.

Microsatellite Analysis

Microsatellite markers used in this study are shown in Figure 1. The sequences and location of the markers were obtained from the Genome Database (<http://gdbwww.gdb.org/gdb/gdbtop.html>) and from the Whitehead Institute for Biomedical Research (<http://www-genome.wi.mit.edu/>). Primer sequences of the tetranucleotide repeat tetra30 were described by Liu et al (2000). The precise location of microsatellites at and around the *LRP1B* gene was obtained from the sequence segments NT_029242.1 and NT_005129.6, the latter containing the *LRP1B* gene (<http://ncbi.nlm.nih.gov>). Matched normal/tumor DNA samples were amplified in 10- μ l reactions with 50 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 200 μ M each dNTP, 5 pmol Cy5-labeled forward primer, 5 pmol reverse primer, and 0.5 U *Taq* DNA polymerase (Gibco BRL, Eggenstein, Germany). After 2 minutes of denaturation at 94° C, the PCR mixes were subjected to the following conditions: 40 seconds at 94° C, 30 seconds at 55° C, and 40 seconds at 72° C for 28 cycles, with a delayed last elongation step for 10 minutes at 72° C in a PTC200 thermal cycler (MJ Research, Watertown, Massachusetts). Before loading, 20 μ l stop solution of 50 mM EDTA and 5 mg/ml Dextran Blue 2000 in 100% deionized formamide were added and the samples were denatured at 95° C for 2 minutes and immediately cooled on ice for 1 minute. Analysis was carried out on an automated DNA analysis system (ALFexpressII, Amersham/Pharmacia Biotech, Freiburg, Germany). The 6% denaturing polyacrylamide gels (acrylamide:bisacrylamide = 19:1) were run at 400 V, 55 mA, 30 W in 1xTBE buffer at a constant gel temperature of 55° C. The collected raw data were evaluated using the Fragment Manager (FM 1.2) software (Amersham/Pharmacia Biotech).

Allelic changes were evaluated according to our score system. Briefly, the complete lack of signal at one allele indicates that each tumor cell has lost one allele and that the tumor DNA is not contaminated with normal DNA. This change is scored as 4. The reduction of signal of one allele to approximately 50% of the corresponding allele in normal tissue was scored as 2, which may correspond to the loss of one allele in approximately 50% of the tumor cell population or to a trisomy, eg, duplication of one allele resulting in a 1:2 allelic ratio. Retention of the normal allelic status in the

tumor cells was scored as 0. Allelic status between 0 and 2 is scored as 1, whereas status between 2 and 4 is scored as 3. Because minimal differences between signals at alleles in normal and tumor tissues may occur due to PCR conditions, we did not evaluate a single score 1 as an allelic imbalance. In cases where most loci were scored 4 along the entire chromosome, the remaining few score 3s were changed to score 4. Thus, at the final evaluation we only used scores 4, 2, and 0. We detected score 4 at least once in each tumor, thereby asserting the purity of the analyzed tumor samples (not shown).

RT-PCR Analysis of the Genes *LRP1B* and *CUL3*

Total RNA was extracted from urothelial carcinomas of the bladder and normal bladder epithelium after their homogenization with Trizol (Life Technologies, Rockville, Maryland) solution according to the manufacturer's instructions. The integrity of the RNA was checked on a 1% formaldehyde/agarose gel. One microgram of total RNA was reverse transcribed for 2 hours at 42° C in 10- μ l volume using 200 units of MMLV-RT (Promega, Mannheim, Germany). After heat-inactivating the enzyme for 5 minutes at 94° C, 10 μ l RNase-free dH₂O were added to the cDNA solutions. Two microliters of cDNA were used as template for each subsequent 20 μ l PCR-reaction. Exon primers 1, 8, 10, and 29 of the *LRP1B* gene and the control *LRP1* primers were used, as described by Liu et al (2000). The primers for *CUL3* were: CAA CAT CCC ACC AGC ACC AAG (*CUL3*-F) and ACT GCT GAG TTA CCT CCG CTA C (*CUL3*-R). The PCR program consisted of an initial denaturation step for 2 minutes at 94° C, and then by 40 cycles of 1 minute at 94° C, 1 minute at 68° C, and 2 minutes at 72° C, finishing with 10 minutes at 72° C. For exon 29 the annealing temperature was reduced to 64° C and for *CUL3* to 60° C. Ten microliters of the PCR products were run on a 1% agarose gel and stained with ethidium bromide.

CGH

DNA of a healthy male donor (reference DNA) was labeled with digoxigenin-11-dUTP, and DNA from 18 UCs were labeled with biotin-16-dUTP (Roche Diagnostics, Mannheim, Germany) using standard nick translation protocols (Lichter and Cremer, 1992). CGH analysis was performed as described previously with minor modifications (du Manoir et al, 1993). Image acquisition, processing, and evaluation were performed using a Leica DM RXA RF8 epifluorescence microscope (Leica, Bensheim, Germany) equipped with a Sensys CCD camera (Photometrics, Tucson, Arizona, Kodak KAF 1400 chip) controlled by the Leica Q-FISH software (Leica Microsystems Imaging Solutions, Cambridge, United Kingdom). Three-color images, green for the tumor DNA, red for the reference DNA, and blue for the DAPI counterstain were acquired from 10 metaphases per sample. Images were processed using the Leica Q-CGH software. The

threshold values for detection of genomic imbalances were 0.75 for losses and 1.25 for gains, respectively.

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