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Target Genes of Recurrent Chromosomal Amplification and Deletion in Urothelial Carcinoma

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Abstract. *Background: Urothelial carcinoma (UC) is characterized by multiple recurrent chromosomal changes on a background of increasing genomic instability. To define target genes of recurrent deletions and amplifications, we explored which gene alterations are common in UC, in two recently established cell lines, BC44 and BC61. Materials and Methods: Genes located in regions of gain or deletion in the cell lines were identified by array comparative genomic hybridization (aCGH). Six published microarray datasets were analyzed for genes differentially expressed between urothelial tumor vs. normal tissues. Gene expression and chromosomal changes were compared in BC61 cells. Results: The cell lines share homozygous deletions at 9p21 around CDKN2A and amplifications at 11q13.2 around CCND1. In both cell lines 11 genes were commonly lost and 115 gained. Across UC in general, 230 genes were expressed stronger and 349 weaker; a subset displaying corresponding genetic changes in the cell lines. The commonly affected subset contains well-investigated genes like E2F1 and CCNE1, but also several genes not yet sufficiently investigated in UC. Discussion: Our approach highlights genes involved in cell cycle regulation, apoptosis and signal transduction as commonly deregulated across UC. Nevertheless, many chromosomal regions undergoing recurrent changes harbor several commonly deregulated genes that may act jointly in UC development and progression.*

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Bladder cancer is a notoriously heterogeneous disease. The major histological type in most countries is urothelial carcinoma (UC), which can be grossly categorized into papillary and invasive subtypes. The second most frequent type, squamous cell carcinoma, is typically associated with chronic inflammation and is more prevalent in countries with schistosomiasis infections. Further rarer types exist, such as adenocarcinoma of the bladder. Unfortunately, the above categories are not always distinct. For instance, many UC contain morphologically evident regions with squamous differentiation or express molecular markers or epidermal differentiation. Likewise, high-grade papillary tumors may progress to invasive urothelial carcinomas (1). A major issue in bladder cancer research is therefore defining the genetic and epigenetic changes shared by all histological subtypes as well as those that are characteristic for each subtype in order to provide a basis for diagnosis, monitoring and treatment. Comprehensive studies on gene mutations, copy number alterations, gene expression changes and epigenetic disturbances in the various subtypes of bladder cancer are underway or have already been published and their evaluation may help resolve these issues (2-8).

Cell lines are an important tool for the study of bladder cancer (9). Ideally, one would like to have cell line panels at hand that represent all subtypes of bladder cancer and contain all relevant molecular alterations. There is indeed a large number of well-characterized cell lines for invasive urothelial cancers with typical genetic changes occurring in these tumors, whereas cell lines from papillary urothelial carcinoma are scarce. Assuming that the low number of cell lines from papillary tumors may be due to the inability of cancer cells to survive and grow in standard culture media, we have cultured cells from papillary urothelial cancers using conditions similar to those for normal urothelial cells, which involve low-calcium, and defined growth factors

(10). Indeed, most primary tumor cultures grew for a few passages under these conditions and two novel cell lines, BC44 and BC61, were derived. BC61 was established from a non-invasive, moderately differentiated (pTaG2) papillary tumor. Upon detailed molecular analysis, the cell line revealed typical characteristics of papillary tumors such as an oncogenic *FGFR3* mutation, deletion of *CDKN2A* and wild-type *TP53*. Nevertheless, karyotyping revealed chromosomal changes indicative of a progressive papillary cancer (11). BC44 was derived from a papillary protrusion of an advanced stage bladder cancer displaying mixed UC and squamous cell carcinoma morphology. Typical of invasive bladder cancers, *FGFR3* was not mutated and *TP53* was inactivated, albeit by an unusual mechanism. Also typical of invasive cancers, the karyotype was aneuploid (11).

We wondered which of the chromosomal alterations shared by these two bladder cancer cell lines might be relevant across bladder cancer in general. Therefore, we identified common regions of copy number gains and losses between the two cell lines and searched for genes located in these regions that show frequent expression changes in bladder cancer overall, by using published microarray expression data. Indeed, in addition to known oncogenes and tumor suppressors in bladder cancer, this analysis revealed a number of chromosomal regions and candidates that may not yet have received sufficient attention in this cancer type.

Materials and Methods

Cell lines and cell culture. The BC44 and BC61 cell lines were derived from primary cultures established as described (10). Cellular and molecular characteristics of these cell lines have been described (11). In brief, both cell lines were routinely maintained in Epilife medium supplemented with 0.25 ng/ml epidermal growth factor (EGF), 12.5 μg/ml bovine pituitary extract (all purchased from Cascade Biologics, Darmstadt, Germany), 1% non-essential amino acids (Invitrogen, Darmstadt, Germany), 1% ITS mix (Invitrogen, Darmstadt, Germany), 3 mM glycine and 10% fibroblast-conditioned medium on a collagen IV matrix (Sigma, Munich, Germany). Cells were passaged twice weekly at a 1:3 ratio using accutase (PAA, Cölbe, Germany) for detachment. Primary urothelial cells (UP) were prepared from ureters after nephrectomy and were routinely maintained in keratinocyte serum-free medium (KSFM, Gibco, Darmstadt, Germany) supplemented with 25 μg/ml bovine pituitary extract and 2.5 ng/ml EGF (12).

DNA and RNA extraction. Total genomic DNA was isolated from sub-confluent cell cultures using the Blood and Cell Culture DNA Midi Kit (Qiagen, Hilden, Germany). Total RNA was isolated from sub-confluent cell cultures using the RNA Mini or Micro Kit (Qiagen).

CGH-array and expression microarray hybridization and evaluation. Array comparative genomic hybridization (aCGH) was performed using an array of 60mer DNA-oligonucleotide probes with a median overall probe spacing of 13 kb (SurePrint G3 Human CGH Microarray 180k, Agilent Technologies, Palo Alto, CA, USA) as described (11) essentially according to the protocol of the manufacturer (Protocol 6.2.1, December 2010, Agilent Technologies). The hybridization patterns were analyzed using a microarray scanner (DNA Microarray Scanner with SureScan High-Resolution Technology, Agilent Technologies, an array feature extraction program (Feature Extraction Software 10.7.1.1, Agilent Technologies, and a data evaluation software package (Genomic workbench 6.5, Agilent Technologies). Deletions and gains were recorded if the Cy5/Cy3- fluorescence ratio exceeded ±0.5 $\log_2$ in one probe. Deletions were considered as homozygous if the Cy5/Cy3 fluorescence ratio exceeding -1 $\log_2$ in at least five consecutive probes. Gains were defined as amplifications if the Cy5/Cy3 fluorescence ratio exceeded +2 $\log_2$ in at least five consecutive probes. As basic adjustment, the ADM-2 algorithm of the data evaluation software was used with a threshold of 6.0.

For gene expression analysis by microarray, total RNA preparations were checked for RNA integrity using the Agilent 2100 Bioanalyzer. All samples in this study showed high quality RNA Integrity Numbers (RIN 10). RNA was quantified by photometric Nanodrop measurement. Synthesis of cDNA and subsequent biotin labeling of cRNA was performed according to the manufacturer's protocol (3' IVT Express Kit; Affymetrix, Inc., City, Country). Briefly, 100 ng of total RNA were converted to cDNA, followed by *in vitro* transcription and biotin labeling of aRNA. Fragmentation labeled aRNA was hybridized to Affymetrix PrimeViewTM Human Gene Expression Microarrays for 16 h at 45˚C, stained by a streptavidin/phycoerythrin conjugate and scanned as described in the manufacturer's protocol.

Data analyses on Affymetrix CEL files were conducted with GeneSpring GX software (Vers. 12.5; Agilent Technologies). Probes within each probeset were summarized by RMA after quantile normalization of probe level signal intensities across all samples to reduce inter-array variability (13). Input data pre-processing was concluded by baseline transformation to the median of all samples. After grouping of samples according to their respective experimental condition (UP236 vs BC61, four replicates each) a given probeset had to be expressed above background (*i.e.* fluorescence signal of a probeset was detected within the 20th and 100th percentiles of the raw signal distribution of a given array) in all four replicates in at least one of two conditions to be further analyzed in pairwise comparisons. In keeping with the analysis below, differential gene expression was statistically determined by moderated *t*-test, using RStudio Bioconductor package limma (14, 15). Resulting *p*-values were corrected for multiple testing by the Bonferroni method (16). The significance threshold was set to $p(\text{corr})=0.05$. All genes are named according to the official gene symbols.

Gene expression analyses of public datasets. The six datasets used were downloaded from Gene Expression Omnibus (GEO) and European Bioinformatics Institute (EBI). Information about these datasets (17-23) is listed in Table I. All genes were investigated for differential expression of urothelial tumor *vs*. normal tissues by a moderated *t*-test, using the R package limma (14, 15). Genes showing nominal *p*-values <0.05 in at least four datasets were considered as "commonly altered in UC". Comparisons with pTa and ≥pT2 tumor tissues were performed analogously. All genes are named according to the official gene symbols.

Table I. *Public microarray expression datasets used in our analysis. References are given in the first row. The Geo Accession codes in the second row identify the datasets in the Gene Expression Omnibus (GEO) and European Bioinformatics Institute (EBI) databases. Total sample numbers with the distribution between normal and cancer tissues are listed in row three. The last row gives the microarray platforms used.*

Authors	Mangueal *et al*. (17)	Lee *et al*. (18, 19)	Dyrskjot *et al*. (20)	Lindgren *et al*. (21)	Stransky *et al*. (22)	Hansel *et al*. (23)
Geo Accession	GSE7476	GSE13507	GDS1479	GSE19915	E-TABM-147	GSE24152
Samples (normal/cancer)	12 (3/9)	265 (68/188)	60 (9/51)	156 (12/144)	57 (5/52)	17 (7/10)
Platform	Affymetrix Human Genome U133 Plus 2.0 Array (HG-U133_Plus_2)	Illumina human-6 v2.0 expression beadchip	Affymetrix Human Genome U133A Array (HG-U133A)	Swegene Human 27K RAP UniGene188 array	Affymetrix HG-U95A and HG-U95Av2 microarrays	Affymetrix Human Genome U133 Plus 2.0 Array (Hs_ENTREZG_10)

Table II. *Homozygous deletions and amplifications in BC44. Homozygous deletions (negative values) and amplifications (positive values) in the BC44 genome identified by aCGH. Chromosomes, cytobands and location of the starting base of the first and last affected probe (hg version 19) are given as well as the number of affected probes. The last column gives the average log ratio of the copy number changes.*

Chr	Cytoband	Start	Stop	#Probes	Amplification/Deletion
chr11	q13.2 - q13.3	68336631	70367700	125	2,51
chr3	p12.3	75959654	76218045	21	–3,62
chr7	q31.1	110310931	110421809	10	–1,30
chr8	p23.3-p21.2	176814	24168932	1369	–1,23
chr8	p23.3-p23.2	605784	6079761	329	–1,34
chr8	q11.21	50241670	51310838	54	–1,32
chr9	p21.3	21902814	21967607	6	–4,58
chr9	p21.3	21902814	23818045	83	–3,19
chr9	p21.3	21968041	22008655	6	–1,86
chr9	p21.3	22356407	22606381	10	–4,22
chr9	p21.3	23042305	23257394	8	–4,25
chr9	p21.3	23569089	23676057	7	–4,33
chr9	p21.3	23736783	23818045	7	–4,75
chr15	q11.1-q11.2	20432851	22558756	55	–1,00
chrX	q21.33	96343280	96449666	9	–2,32
chrX	q28	154783535	154908471	8	–2,05

Results

Detailed analysis of chromosomal changes in BC44 and BC61. The karyotypes and gross copy number changes of the BC44 and BC61 cell lines have been previously described (11). In brief, BC44 is aneuploid with a pseudotriploid (female) genome displaying major rearrangements of several chromosomes including chromosomes 1, 3, 4, 6, 7, 8, 16, 17, 18 and 19. BC61 has a tetraploid (male) karyotype with a more limited number of chromosomal changes, most of which are typical of progressive papillary urothelial carcinomas, such as loss of chromosome 9 and chromosome 11, isochromosome 5p and gain of chromosome 20. We have now performed a detailed analysis of the aCGH data for these cell lines in order to define the genes affected by amplifications or deletions in either cell line as well as in both.

Applying stringent criteria, presumable homozygous deletions in BC44 are detected at 3p12.3, 7q31.1, 8q11.21, 8p23.3 - p23.2, 9p21.3, 15q11.1 - q11.2, Xq28 and Xq21.33 (Table II). As commonly observed in cancer, many homozygous deletions contain no or few protein-coding genes (24). The prime exception is the 9p21 deletion (Figure 1A) including the tumor suppressor genes *CDKN2A* and *CDKN2B*, the most frequent target of homozygous deletions across all human cancers (25). In BC44 this deletion extends across 1.94 Mbp. A large deletion of the terminal 8p region encompassing a large number of genes, including several tumor suppressor candidates, is actually rather monosomic in the approximately triploid genome. The most clear-cut amplification unit in BC44 involves a region at 11q13.2 - q13.3 of approximately 2.03 Mbp centered around the *CCND1* gene encoding Cyclin D1 (Figure 1B, Table II).

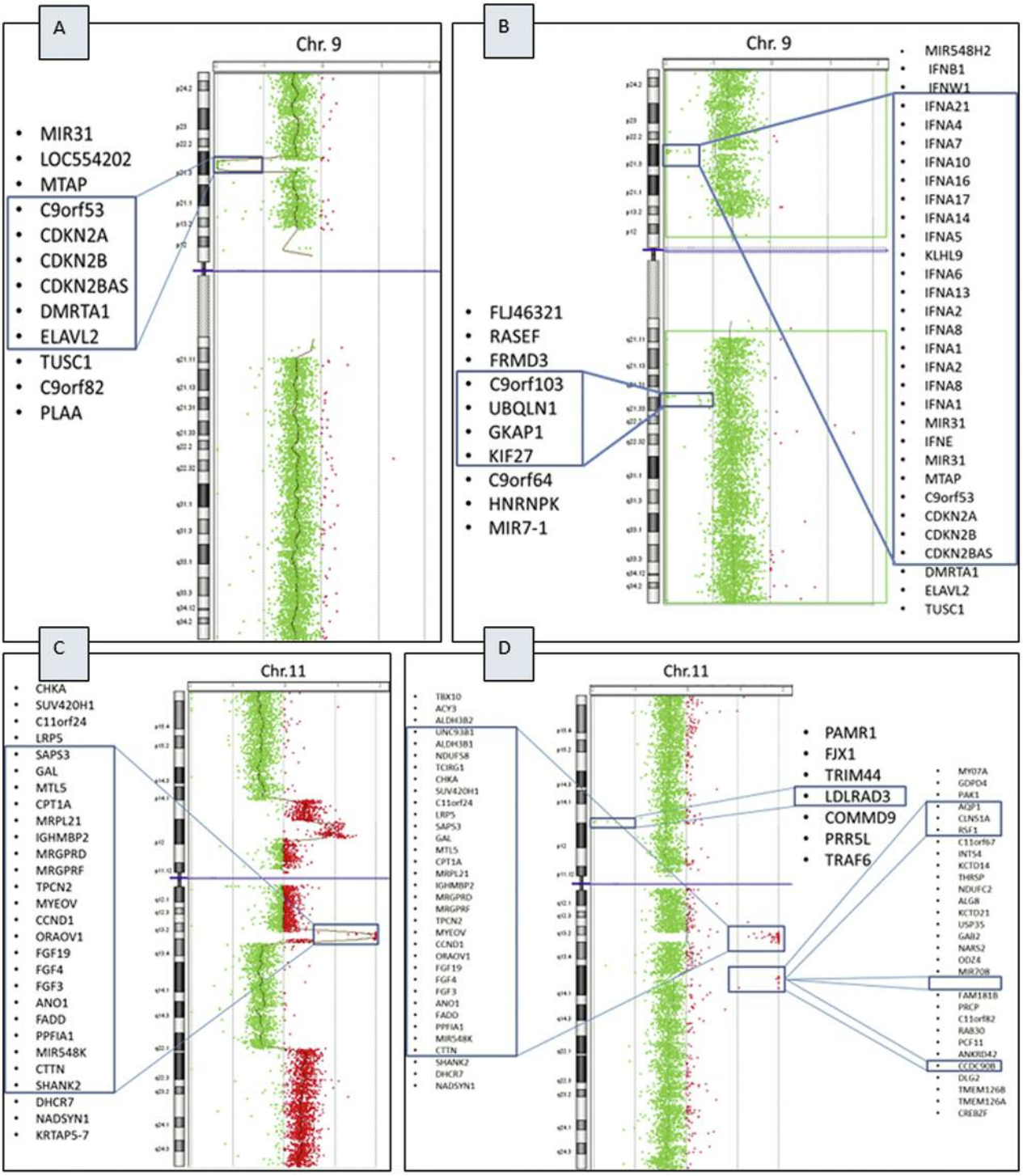

Figure 1. *Detailed structure of homozygous deletions and amplifications on chromosome 9 and 11 in BC44 and BC66. Affected genes are indicated within the blue boxes and the next unaffected adjacent genes are marked outside each box. A: Chromosome 9 in BC44. B: Chromosome 9 in BC61. C: Chromosome 11 in BC44. D: Chromosome 11 in BC61.*

Table III. *Homozygous deletions and amplifications in BC61. Homozygous deletions (negative values) and amplifications (positive values) in the BC44 genome identified by aCGH. Chromosomes, cytobands and location of the starting base of the first and last affected probe (hg version19) are given as well as the number of affected probes. The last column gives the average log ratio of the copy number changes.*

Chr	Cytoband	Start	Stop	#Probes	Amplification/Deletion
chr11	q13.2 - q13.3	67768754	68492834	58	2,33
chr11	q13.3	68492895	70298431	106	2,33
chr11	q14.1	77303540	77386325	5	3,00
chr11	q14.1	80215689	80714883	12	2,47
chr11	q14.1	83277858	83429018	12	2,41
chr12	q15	69298660	69546488	10	2,42
chr12	q15	69546489	69970431	36	3,09
chr12	q15	69970432	70341007	22	2,42
chr1	q31.3-32.1	198618159	198817875	16	–1,04
chr2	q21.2	133194005	133355824	11	–2,52
chr3	p26.1	7236242	7639780	30	–1,00
chr3	p26.1	7086321	7236181	12	–1,00
chr3	p26.1	7668960	8306181	28	–1,00
chr3	q13.31	116229141	116831957	29	–1,09
chr4	q13.3	71162798	71283216	10	–1,26
chr9	p21.3	21282036	21409799	9	–3,00
chr9	p21.3	21454717	21512444	9	–4,72
chr9	p21.3	21520414	21708370	11	–3,00
chr9	p21.3	21708371	21872590	10	–4,00
chr9	p21.3	21902814	21967607	6	–4,53
chr9	p21.3	21968041	22009029	7	–1,93
chr9	p21.3	22086858	22356466	8	–3,00
chr9	q21.32	86276560	86309360	5	–3,81
chr9	q21.32	86309361	86468944	11	–2,36
chr11	p13	35941265	36043690	8	–3,95
chrX	q26.2	132513848	132570434	6	–3,53

By the same criteria, homozygous deletions in BC61 are detected at 1q31.3 - q32.1, 2q21.2, 3q13.31, 3p26.1, 4q13.3, 9p21.3, 11p13 and Xq26.2; amplifications are found at 11q13.2 – q13.3, 11q14.1 and 12q15 (Table III). Notably, despite its overall less aberrant karyotype, the number of focal deletions and amplifications is not appreciably lower in BC61 than in BC44 (Table III). In addition, all regions with homozygous deletions actually contain protein-coding genes. Notably, the deletion at 9p21.3 of about 1.19 Mbp and the amplification at 11q13.1-13.2 of about 2.53 Mbp are common to both cell lines, albeit differing in extension (Figure 1C, D). The additional amplification unit at 12q15 in BC61 is particularly interesting as it contains mouse double minute 2 homolog (MDM2).

We then applied less stringent criteria to identify genes affected by chromosomal gains and losses in the two cell lines. This procedure yielded 1075 (BC61) and 305 (BC44) genes, respectively, subject to loss and 551 (BC61) and 2001 (BC44) genes, respectively, subject to gains. Of these, 11 genes were lost and 115 were gained in both cell lines (Table IV).

Comparison of chromosomal changes in BC44 and BC61 to common expression changes in UC. In order to investigate whether genes affected by copy number changes in the two cell lines are regularly deregulated in bladder cancers, 6 published expression microarray datasets were analyzed (Table I). In the first step, all genes were selected from each individual array that showed significantly stronger or lower expression in tumors compared to normal tissues, without adjustment for multiple testing. Instead, in the second step we considered those genes that were significantly changed ($p<0.05$) in at least 4/6 arrays as "commonly deregulated in bladder cancer". Based on all platforms, 349 genes were commonly down-regulated and 230 genes were commonly up-regulated. Of note, the Lindgren *et al*. (50) study used a different array platform. Omitting this dataset therefore leads to a larger set of commonly deregulated genes.

According to an analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 bioinformatics tool (26, 27), the genes commonly down-regulated in UC were particularly over-represented in the Gene Ontology groups "response to endogenous stimulus" ($p_{adj}<10^{-5}$) and in its subgroup "response to steroid hormone stimulus" ($p_{adj}<10^{-5}$). A disproportionate fraction of the down-regulated genes encodes plasma membrane proteins

Table IV. *Genes commonly affected by deletions or gains in BC44 and BC61. Genes commonly affected by deletions or gains in BC61 and BC44 identified by aCGH. The columns list the genes exhibiting concordant changes in both cell lines.*

Gain						Amplification	Deletion	Homozygous Deletion
ACSS2	CBFA2T2	EFCAB8	LOC100130264	PET117	SNTA1	ANO1	AGBL1	C9orf53
AHCY	CCND1	EIF2S2	LOC284805	PIGU	SNX5	CCND1	C9orf53	CDKN2A
ANO1	CDK5RAP1	EIF6	MAP1LC3A	PLAGL2	SUN5	CPT1A	CDKN2A	CDKN2B
ASIP	CHMP4B	FADD	MAPRE1	PLK1S1	TM9SF4	CTTN	CDKN2B	CDKN2BAS
ASXL1	COMMD7	FAM83C	MIR644	PLUNC	TP53INP2	FADD	CDKN2BAS	
BASE	COX4I2	FGF19	MMP24	POFUT1	TPCN2	FGF19	CNNM2	
BCL2L1	CPT1A	FGF3	MRGPRF	POLR3F	TPX2	FGF3	ELAVL2	
BPIL1	CRNKL1	FGF4	MRPL21	PPFIA1	TRPC4AP	FGF4	EXD3	
BPIL3	CSRP2BP	GAL	MTL5	PROCR	TTLL8	GAL	SOCS6	
C20orf112	CTTN	GGT7	MYEOV	PXMP4	TTLL9	IGHMBP2	SYK	
C20orf114	DEFB118	GRM7	MYH7B	RALGAPA2	WNK2	MIR548K	XPNPEP1	
C20orf12	DEFB119	GSS	MYLK2	RALY	XKR7	MRGPRD		
C20orf160	DEFB121	HCK	NAA20	RBBP9	XRN2	MRGPRF		
C20orf185	DEFB123	HM13	NCOA6	REM1	ZNF133	MRPL21		
C20orf186	DNMT3B	HSPC072	NECAB3	RIN2	ZNF341	MTL5		
C20orf26	DTD1	ID1	NKX2-2	RORA		MYEOV		
C20orf70	DUSP15	IGHMBP2	ORAOV1	SAPS3		ORAOV1		
C20orf71	DYNLRB1	INSM1	OVOL2	SEC23B		PPFIA1		
C20orf72	E2F1	ITCH	PAX1	SHANK2		SAPS3		
C20orf79	EDEM2	KIF3B	PDRG1	SLC24A3		TPCN2		

($p_{adj}=10^{-6}$). In addition, several under-expressed genes regulate cell death or apoptosis ($p_{adj}=10^{-2}$). The functions of the commonly up-regulated genes were less diverse. Interestingly, however, almost all of the significantly enriched GO groups related to cell cycle, mitosis, DNA replication and repair ($p_{adj}=10^{-6}$).

Comparing the genes "commonly deregulated in bladder cancer" to those accordingly lost or gained in BC44 or BC61 gave sizable sets of overlap, shown in Table V.

We then performed the same analysis for the microarray datasets considering only differences between muscle-invasive cancers (≥pT2) and normal tissues or considering only differences between non-invasive papillary tumors (pTa) and normal tissues. Twenty-three genes remained significant across all analyses (Table VI). These genes are predicted to become gradually up- or down-regulated during UC progression.

Comparison of genomic and expression changes in BC61. In a final series of analyses, we addressed the question of how numerical chromosomal changes in BC61 relate to changes in gene expression. For that purpose, an expression microarray analysis was performed comparing BC61 with normal urothelial cells in primary culture (UP), which have a normal genome. Of note, these cells proliferate at a similar rate as UC cell lines (28). Four independent RNA preparations from each cell type were used and applied to Affymetrix PrimeView™ Human Gene Expression microarrays.

Following normalization and centering of RNA expression data, 39,367 out of 49,394 probes on the array were evaluable. After Bonferroni-adjustment for multiple testing, 8,755 probes were differentially expressed at $p<0.05$ by moderated *t*-test. Of these, 4,558 probes corresponding to 2,906 genes were more strongly expressed in BC61 and 4,558 probes corresponding to 2,614 genes were more strongly expressed in UP. The 100 genes with the highest fold changes are listed in table VII.

The differentially expressed genes were analysed for systematic differences using the DAVID bioinformatics platform (26, 27). The genes with lower expression in BC61 were highly significantly localized on chromosomes 9 (230 genes), 5 (171 genes), 10 (184 genes) and 16p13.3 (38 genes). In functional terms, they were particularly enriched in genes involved in RNA biogenesis and metabolism, including cellular and mitochondrial ribosomal biogenesis. This enrichment could partly, but not completely, be explained by the localization of the relevant genes in chromosomal regions lost in BC61. Among the up-regulated genes, 187 were localized on chromosome 20, making localization on this chromosome a striking characteristic. In functional terms, transcription factors, activators and repressors, and especially zinc finger proteins were significantly over-represented.

Table V. *Genes within chromosomal regions of deletions or gains in BC44 or BC61 with differential expression between tumor and normal tissues in six publicly available RNA expression datasets. Deletions and gains were identified by aCGH. RNA expression: up- or down- expressed genes in urothelial cancer tissue, with a p-value <0.05 (moderated T-test) in the comparison of urothelial cancer of all stages with normal urothelial tissue. The underlined genes are also differentially expressed in BC61 compared to normal urothelial cells.*

Deleted in BC61		Deleted in BC44		Gained in BC61		Gained in BC44	
Cytoband	Gene	Cytoband	Gene	Cytoband	Gene	Cytoband	Gene
chr1q31.3	PTPRC	chr7q36	PTPRN2	chr11q13.3	PPFIA1	chr1q21	MTX1
chr3p26.1	GRM7	chr7q36	RHEB	chr20 p13	SNRPB	chr1q21	VPS72
chr9p13.2	ALDH1B1	chr7q36.1	KCNH2	chr20q11.2	TPX2	chr1q21.2	APH1A
chr9p13.3	NFX1	chr7q36.1	GIMAP5	chr20q11.21	BCL2L1	chr1q21.3	FLAD1
chr9p22.3	PSIP1	chr8p21.2	ADAM28	chr20q11.22	ERGIC3	chr1q21.3	LASS2
chr9p24.1	NFIB	chr8p21.2	HR	chr20q11.23	RPN2	chr1q21.3	S100A11
chr9p24.3	SMARCA2	chr8p21.2	PHYHIP	chr20q13.13	CSE1L	chr1q21.3	C1orf77
chr9q13-q21	ZFAND5	chr8p21.3	BMP1	chr20q13.2	ZNF217	chr1q21-q22	RUSC1
chr9q21	GNAQ	chr8p21.3	PPP3CC	chr20q13.3	PTK6	chr1q21-q23	SSR2
chr9q21.2	VPS13A	chr8p21.3	SORBS3	chr20q13.31	RAE1	chr1q22	FDPS
chr9q21.31	TLE4	chr8p22	PCM1			chr1q23.2	COPA
chr9q21.33	NTRK2	chr8p22	SLC7A2			chr1q23.2	NCSTN
chr9q22	ROR2	chr8p23.3	KBTBD11			chr1q23.2	PEX19
chr9q22.3	CDC14B	chr16q23.1	WWOX			chr1q23.3	B4GALT3
chr9q22.3	TMOD1					chr1q24.1	TMCO1
chr9q22.33	TGFBR1					chr1q24.1	UCK2
chr9q33	GSN					chr1q32.1	ELF3
chr9q33.2	CDK5RAP2					chr1q32.2	CD46
chr9q33.3	GOLGA1					chr1q32-q41	CENPF
chr9q33-q34	TRAF1					chr1q42.12	PARP1
chr9q34.1	C9orf16					chr5p13.1	NUP155
chr9q34.11	SPTAN1					chr5p13.2	SKP2
chr9q34.11	USP20					chr5p15.2	DAP
chr10q23.3	PTEN					chr5p15.33	TRIP13
chr10q23.31	FAS					chr10q21.2	CDC2
chr10q23-q24	DNTT					chr11q13.3	PPFIA1
chr10q24.32	ARL3					chr12p11.21	FAM60A
chr10q24.32	BTRC					chr19q12	POP4
chr10q25.2	ADD3					chr19q13.1	ZNF146
chr16p13.3	UBE2I					chr19q13.12	FXYD3
						chr19q13.12	LSR
						chr19q13.2	MRPS12
						chr19q13.2	PAFAH1B3
						chr19q13.2	SPINT2
						chr19q13.2-q13.3	LIG1
						chr19q13.3	RUVBL2
						chr19q13.32	AP2S1
						chr19q13.33	KDELR1
						chr19q13.43	UBE2M
						chr19q13.43	ZNF544
						chr20q11.2	TPX2
						chr20q11.21	BCL2L1

This analysis indicates that differences in gene expression between BC61 and normal urothelial cells are dominated, albeit not fully determined by chromosomal gains and losses. For a systematic analysis of the relation between chromosomal changes and gene expression changes, the gene lists obtained by aCGH and expression microarray analysis were compared. Indeed, whereas 310 genes with lower expression in BC61 had lower copy numbers, only 30 had increased copy numbers. Conversely, 237 genes with higher expression in BC61 had increased copy numbers, but only 48 had lower copy numbers. However, it should be noted that a large number of genes showed expression changes without copy number changes and conversely, a large number of genes with copy number changes remained steady in expression.

Table VI. *Genes affected by deletions and gains in BC44 or BC61 with differential expression across all tumor stages in the six public RNA expression files. These genes are a subset of those in Table IV. Their expression differs significantly not only between normal and cancer tissues, but also between normal tissue and pTa tumors as well as between normal tissue and muscle-invasive carcinomas. The underlined genes are additionally differentially expressed in BC61.*

Deleted in BC61		Deleted in BC44		Gained in BC61		Gained in BC44	
Cytoband	Gene	Cytoband	Gene	Cytoband	Gene	Cytoband	Gene
chr10 q23.31	FAS	chr7 q36.1	KCNH2	chr11q13.3	PPFIA1	chr1 q21	VPS72
chr10 q25.2	ADD3	chr8 p21.3	PPP3CC	chr20 p13	SNRPB	chr1 q21	MTX1
chr9 q22	ROR2	chr16 q23.1	WWOX	chr20 q11.2	TPX2	chr1 q21.3	FLAD1
chr9 q33	GSN			chr20 q11.23	RPN2	chr1 q21-q22	RUSC1
						chr1 q22-q23	NCSTN
						chr1 q42.12	PARP1
						chr5 p13.1	NUP155
						chr10 q21.2	CDC2
						chr11 q13.3	PPFIA1
						chr19 q13.2	PAFAH1B3
						chr19 q13.12	LSR
						chr20 q11.2	TPX2

Discussion

The technical progress in next-generation sequencing has allowed large-scale analyses that are expected to lead to a comprehensive catalogue of genomic changes in all human cancers in the near future (8). In the context of these projects, several consortia are addressing urothelial cancer. Among others, two papers by a Chinese consortium (5, 6) delineate mutations and copy number changes in a large (99 cases) series of UC. A comprehensive analysis of invasive UC in US-American patients has recently been published by the TCGA consortium(8). In addition, several European groups have published large sets of expression data and copy number changes (3, 4), some of which have been used in the present analysis.

The task at hand in bladder cancer research is now the interpretation of this huge amount of data, with the ultimate aims of understanding the biological mechanisms driving the development and progression of this common cancer, and of translating the acquired knowledge into improved diagnostic and therapeutic procedures. One important step towards this ultimate goal, is the identification of relevant genes targeted by copy number changes, which often encompass large regions of the genome. Our findings in two newly established bladder cancer cell lines can serve as an example to this end.

Despite their quite different origin, both cell lines had in common one amplification at 11q13 and a homozygous deletion at 9p21 that is thought to primarily target the established tumor suppressor genes *CDKN2A* and *CDKN2B*. Of note, other genes in the region may also be relevant, *e.g.* *ELAVL2* [3]. The amplification unit at 11q around the *CCND1* gene, likewise, is actually quite large and affects several genes, some of which are indeed overexpressed in BC61. Regardless, the coexistence of these two alterations is a bit puzzling since the products of *CDKN2A*, *CDKN2B* ($p16^{INK4A}$ and $p15^{INK4B}$) and of *CCND1* (Cyclin D1) act in the same pathway, namely regulation of CDK4/6. Both inactivation of *CDKN2A*, most frequently by homozygous deletion, and amplification of *CCND1* are common alterations in UC. Further common changes in this pathway include amplification and overexpression of *E2F1* and *E2F3*, and loss of pRB1 function through gene mutation and deletion (4, 6, 8, 19). *RB1* mutations are complementary to *CDKN2A*, but are often accompanied by amplification and overexpression of *E2F3*, another apparent redundancy in need of explanation (4, 29). Accordingly, only *E2F1*, but not *E2F3* or *RB1*, showed conspicuous changes in the two new cell lines.

A strong argument can be made that the establishment of bladder cancer cell lines requires the inactivation of the RB1/$p16^{INK4A}$/CDK4 circuit that controls the cell cycle in G1 and may prevent immortalization (30). In a similar fashion, almost all bladder cancer cell lines have defects in p53, mostly by mutations coupled with loss of one allele of the *TP53* gene located at 17p. The concomitant inactivation of both control systems seems to be characteristic of invasive bladder cancers (1, 31). Alternative to mutations of the *TP53* gene itself, p53 function may become compromised by loss of $p14^{ARF1}$, encoded by *CDKN2A* in an alternative reading frame, or by *MDM2* amplification. In the BC44 cell line, p53 is inactivated by an unusual mechanism, present in a small fraction of bladder cancers, which leads to the sole expression of protein p53Δ133 isoforms lacking the amino-terminal transactivating domain (11). The discovery of a MDM2

Table VII. *Top 100 genes expressed more strongly in BC61 or in normal uroepithelial cells (UP) by fold change. All genes were significantly differentially expressed according to Bonferroni-corrected moderate T-test.*

Top 100 genes expressed more strongly in BC61					Top 100 genes expressed more strongly in UP				
CCND2	ALDH7A1	PRSS3	ALOX5AP	FADS2	MAGEA3	HOXC6	CHKA	RASSF2	AKR1B1
STOM	CD200	CA12	CDH13	PLAGL1	MAGEA6	NLRP2	PPP6R3	GBP2	INPP1
KLHL9	CRCT1	LIPG	CCDC8	C16orf5	CALB1	FAM46A	LCN2	PLAC8	RCAN1
MSN	EDNRA	LY6K	CD44	PDCD1LG2	LOXL4	IGFBP3	LY6D	NCOA7	ZNF488
AREG	VIM	RAB31	GPC4	FST	C1R	GPR128	MEIS2	SLC16A7	CXCL3
AREGB	UBQLN1	TFPI2	THBS1	UGT2B7	LUM	SUV420H1	PTCHD4	SLC2A12	CFI
BNC1	EPB41L3	THBS2	ERRFI1	FKBP10	CLEC7A	MDK	GAL3ST4	AKR1B10	MPPED2
AKAP2	TM4SF1	PCGF5	COL4A2	MTAP	ZIC2	CEBPA	NMNAT2	AKR1B15	VTCN1
PALM2-AKAP2	FKBP5	PXDN	DSC3	AUTS2	RNASE1	FRS2	HS6ST3	HTR2C	TGFBR3
PTHLH	TUBB6	GJA1	IGFBP7	XDH	LUZP2	BMP3	EIF1AY	FBLN1	GATA6
SERPINE2	LRRC8C	TNFRSF10D	DCBLD2	CWH43	DDX3Y	LXN	UCA1	COL3A1	MMP12
MT1G	LAMC2	INHBA	XIST	KLRC1	BCAS1	ELF3	YEATS4	RAB3IP	DRAM1
ANGPTL4	LDLR	ZNF826P	DUSP5	KLRC2	PPP2R2B	OLR1	GABRB3	COL5A2	PTPRR
LOC100653217	HMGA2	SLC16A4	DSC2	SQRDL	WNT10A	KCND2	TXLNG2P	PPAP2A	LRP5
NTM	KCNMA1	PRTFDC1	FBLIM1	QPRT	FYB	METTL7A	HCAR2	ITGAM	SLC1A3
PLEK2	HLA-C	C2orf74	GRB10	AHNAK2	AQP11	NMB	HCAR3	EPHA4	C3
LOC100132240	CHST2	KIAA1841	LAMA3	GNG11	BPIFB1	SIM2	TNFAIP2	MMP13	APOBEC3B
MIG7	G0S2	ADRB2	FAM171A1	SLC16A3	IGFBP6	SLC40A1	LOC100509121	PITX2	GABBR1
DKK3	FHL1	TRNP1	FN1	ZBED2	DAPK1	CLNS1A	ERP27	OBSL1	UBD
NT5E	MEST	BNIP3	DBC1	RGMB	BST2	LIMCH1	MDM2	PBX1	HPGD

amplification in BC61 suggests an explanation of how this cell line copes with the presence of wild-type p53. Interestingly, a similar constellation has been reported in the cell line BFTC905, which was derived from a pTa high grade UC (32). Indeed, in the analysis of public microarray datasets, MDM2 overexpression is only nominally significant in the comparison of pTa tumors with normal tissues. Several dedicated studies confirm that MDM2 overexpression is more often associated with pTa and pT1 tumors (4, 31, 33, 34).

Of note, the amplification unit at 12q contains a number of genes in addition to *MDM2* that are overexpressed in BC61. The same is true for the 11q amplification unit. For instance, our analyses revealed *PPFIA1* as a gene with overexpression gradually increasing with UC progression. Coamplification of *PPFIA1* with *CCND1* has previously been noted in other cancer types (35, 36). In addition, the amplified region encodes the *MiR-548k* gene. MicroRNAs are now recognized as important factors in tumor development (37). Our study highlights the genes encoding miR-644 and miR-548k. *MiR-548k* at 11q13 is amplified in both cell lines and its product interacts with many genes (targetScanHuman) that are important for cell metabolism and survival, whereas miR-644 may regulate housekeeping genes like *GAPDH* and could be a confounder of measurements using these as a reference (38). To our knowledge, neither miRNA has been studied in detail in UC to date.

In addition to the 9p21 region, several further homozygous deletions were detected in either BC44 or BC61. In principle, homozygous deletions in a cancer could signal the location of a potent tumor suppressor, but - paradoxically - could alternatively occur at regions that are completely dispensable for tumor cell survival, such as "gene deserts". The latter seems to be the case in BC44, whereas true homozygous deletions in BC61 do contain defined genes. However, none of these was recurrently down-regulated in the microarray analyses and they are, therefore, unlikely to represent a major tumor suppresor gene. The issue is different for the distal part of chromosome 8p, which is strongly under-represented in BC44, but not truly homozygously deleted. This region has long been implicated in UC progression (39). It contains a large number of plausible tumor suppressor candidates, but none has been identified as individually critical in UC. According to our analysis, expression of a number of genes located in 8p21-23.2 (*ADAM28, HR, PHYHIP, BMP1, PPP3CC, SORBS3, PCM1, SLC7A2, KBTBD11*) is commonly diminished. Collectively, our analysis supports the proposition of Williams *et al*. (39) that loss of several genes at 8p may contribute to urothelial carcinogenesis. Accordingly, collaboration of several tumor suppressors at 8p22 has recently been reported to contribute to hepatocarcinogenesis (40).

The published large-scale mutational analyses (6, 8) suggest particular oncogenes or tumor suppressors apparently targeted by other chromosomal changes common to BC44 and BC61. However, many chromosomal changes encompass large regions, as seen prominently in the two cell

lines. In addition, point mutations are not the only indicators of genes involved in carcinogenesis and are, in fact, sometimes difficult to interpret. In general, however, one would expect oncogenes located in a region undergoing gain or amplification to be overexpressed and conversely, tumor suppressors affected by deletions to be down-regulated. The comparison of gene expression and copy number changes in BC61 illustrates this point. Thus, a much higher fraction of genes located in gained chromosomal regions were up-regulated than down-regulated and conversely, a much lower fraction located in regions undergoing loss were down-regulated than up-regulated. However, the majority of genes located in such regions did not show significant or large changes in gene expression. Thus, filtering for genes undergoing expression changes in the expected direction should restrict the number of potential pro- and anti-oncogenic genes within a particular chromosomal region.

Indeed, considering the genes located in regions of gain or loss in the two cell lines (beyond those affected by true amplifications or homozygous deletions) and searching for those that were deregulated in several published microarray datasets, yielded a list of candidates in particular for chromosomal regions that are not as well-explored in UC, albeit commonly affected by copy number changes (Table V). Such regions include 8p for deletions and 1q, 5p, 19q and 20q for gains. In addition, the comparison of published expression data sets and copy number changes in BC44 and BC61 highlighted 23 genes (Table VI) that appeared to become up- or down-regulated in a gradual fashion during UC progression.

Despite many provisions from biological and statistical arguments applying to this sort of analysis, several genes identified are good candidates for further investigation in UC. First, the products of *CDC2* (CDK1) and *SKP2* are both involved in the regulation of cell cycle transition from late G1 to S-phase, like E2F1 discussed above. Indeed, *CDC2* has already been implicated in the progression of UC (41). In contrast, the F-Box protein SKP2, which is involved in the degradation of several important proteins including $p27^{KIP1}$, a crucial antagonist of Cyclin E1, is poorly studied in this cancer type, despite its importance in others (42). Collectively, these findings indicate that the disturbances in the regulation of cell cycle in UC are not limited to the early G1 phase, *i.e.* by changes in Cyclin D1 and $p16^{INK4A}$. Interestingly, the *SKP2* gene is located on chromosome 5p, which is frequently gained in UC, often by formation of i5p with concomitant loss of the long arm. This chromosome arm contains other genes known to contribute to UC, prominently the telomerase gene *TERT*, which may explain why i5p is prevalent in papillary tumors. Conceivably this common gain may target both *TERT* and *SKP2*. The nucleoporin gene *NUP155* at 5p counts among the gradually up-regulated genes.

Secondly, the list contains several genes deleted in common regions of deletion. At 8p21-22, *SORBS3* and *PPP3CC* are particularly notable. SORBS3 encodes Vinexin, which is involved in focal and cell-cell adhesion. So far, it has only been studied in hepatocellular carcinoma (43). *PPP3CC* encodes a regulatory subunit of the calcium-dependent protein phosphatase calcineurin. Calcineurin limits stem cell potential in the epidermis (44), but it is unknown whether it functions in the same manner in the urothelium. Interestingly, down-regulation of calcineurin occurs during bladder hypertrophy (45). Interestingly, as a regulator of non-canonical WNT-signaling, the product of the *ROR2* gene at 9q, commonly and progressively down-regulated in UC, may act upstream of calcineurin (46)

Further genes highlighted on chromosome 9q include *SPTAN1, ZFAND5, GNAQ, TLE4, CDC14B, TMOD1, GSN, CDK5RAP2, TRAF1* and *USP20*, which have not yet been explored in the context of UC. A few, like *NTRK2* and *TGFBR1*, have already been investigated because of their function in regulating GSK3 and TGFβ signaling, respectively (47, 48). Deletions at 9q are among the most frequent chromosomal changes in UC, but neither their targets nor their impact is fully understood despite intense research (31). Overall, it is likely that the deletions target several genes in a synergistic manner. The same argument may even hold for chromosome 10q, where *PTEN* is an established target for deletions that are typically associated with tumor progression (31). Our present analysis highlights *FAS* (also known as *TNFRSF6*), a death receptor known to become progressively dysfunctional in UC and contribute to apoptosis resistance (49, 50) as a further target.

The third group of genes deserving explicit discussion is that on chromosome 20. Gain of chromosome 20 is common in UC, but it is only partly understood which genes are targeted. One interesting candidate is the anti-apoptotic gene *BCL2L1*, for which focal amplifications have been reported recently (8). Two additional chromosome 20 genes identified here are *ERGIC3* and *TPX2*. ERGIC3 is an ER protein implicated in the development and progression of lung cancer (51). *TPX2* (TPX2, microtubule-associated) is commonly overexpressed in UC and the gene is gained in both cell lines. *TPX2* is an oncogene candidate in several cancers, likely acting through its effects on mitosis and the spindle apparatus. There is direct evidence that it contributes to bladder cancer progression (52).

Table VI lists several genes that are becoming progressively deregulated in UC, which have already been linked to phenotypes involved in tumor progression, *KCNH2* (53), *GSN* (54), *LSR* (55), and *FAS* (49), with specific data in UC for the latter three. Furthermore, *NCSTN* and *PARP1* are gained in BC44 and commonly overexpressed in UC. They are located at chromosome 1q, where, in UC 1, q22-24 is often affected by gains or amplifications (4, 56, 57).

NCSTN encodes nicastrin, a component of γ-secretase, which processes the amyloid precursor protein, but also, presumably more relevant to cancer, notch proteins. Notch activation is pro-tumorigenic or tumor-suppressive in a cell-type dependent fashion (58); in UC, there is insufficient data to date (59, 60). *PARP1* encodes the poly (ADP-ribose) polymerase I which influences many cellular processes, but is best known for its function as a DNA repair cofactor and as a therapeutic target (61, 62). Its function in UC certainly deserves detailed investigation. Another such candidate is *WWOX* (WW domain-containing oxidoreductase), which is deleted in BC44 and commonly down-regulated in UC. The gene is located at a fragile site in 16q23.3-24.1 and likely acts as a tumor suppressor in many cancers (63).

Finally, a number of genes at chromosome 19q13 gained in BC44 are up-regulated in UC. Of these, *LSR* and *FXYD3* have been studied. The FXYD3 protein, a regulator of ion transport across cellular membranes, is overexpressed in the majority of UC of the upper genitourinary tract and in high grade bladder cancers (64). The "lipolysis stimulated lipoprotein receptor" encoded by *LSR* is often up-regulated in UC. Its knockdown by siRNA significantly increased the motility and invasion capacity of bladder cancer cells (55). Notably, in breast cancer *LSR* displayed opposite properties (65). These discrepant findings along with the results of our analysis indicate a need for further investigations of LSR in cancer.

In conclusion, our study provides several novel candidate genes in chromosomal regions with recurrent gains or losses in UC in addition to already well-studied genes. In particular, it highlights several regions that have not been subjected to close scrutiny yet, such as 5p or 20q, that obviously deserve even more attention, like 8p. Moreover, our analysis supports the idea that many chromosomal changes in UC target several genes with additive or synergistic effects on tumor development and progression.

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