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Loss of heterozygosity on chromosomes 11 and 17 are markers of recurrence in TCC of the bladder

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Summary Approximately 2/3 of patients diagnosed with superficial transitional cell carcinoma of the urinary bladder (TCC) will recur within 2 years. Loss of chromosome 9 and loss of heterozygosity (LOH) at 9q34 in index TCCs identify a subset of patients at high risk of recurrence. This study explores genetic alterations on chromosomes 4, 8, 11 and 17 as predictors of recurrence. A total of 109 carcinomas were investigated at 26 loci. DNA was extracted from microdissected archival normal/tumour tissue and was analysed for loss of heterozygosity (LOH). Fluorescent PCR was performed and genotyping carried out on a Perkin Elmer ABI377 sequencer. LOH of D11S490 or D17S928 was significantly more frequent in index carcinomas of patients who experienced recurrence compared to those with no recurrence ($P = 0.004$ and 0.019 respectively). These results suggest that loss of these regions is associated with recurrence of TCC. Further investigation is required to identify genes in these regions, which might be responsible for driving recurrence in TCC of the urinary bladder. © 2001 Cancer Research Campaign http://www.bjcancer.com

Keywords: LOH; TCC of the urinary bladder; chromosome 11 and 17

Most (80%) transitional cell carcinomas (TCC) of the urinary bladder are low stage (pTa or pT1) at initial presentation (Van der Meiden, 1999) and may be surgically removed leaving no detectable disease. However, 50–70% of these patients present with a recurrence within 2 years and 20% of the recurrences progress to detrusor muscle invasion. TCCs that invade the muscle cannot be eradicated by endoscopic surgery alone; and 50% of patients with this disease develop metastases and die within 5 years (Adshead et al, 1999).

Currently only a few genetic aberrations are associated with development and progression of TCC of the urinary bladder, e.g. loss of chromosome 9, p53 mutations and gain of *c-myc* (Bartlett et al, 1998; Tsutsumi et al, 1997; Sauter et al, 1995). Such genetic changes may determine the path a TCC will follow (Bartlett et al, 1998; Watters et al, 2000). Several studies demonstrate genetic differences between pTa and pT1 carcinomas (Richter et al, 1997; Simon et al, 1998). These studies suggest that a higher level of genetic abnormalities in pT1 carcinomas increase their likelihood of progression. It is, however, difficult for the pathologist to accurately stage pTa/pT1 carcinomas. Therefore, even if this distinction was proven, identification of the carcinoma suppressor genes that drive TCC recurrence and/or progression would not only increase our understanding of TCC disease development and progression but would also enable us to predict the path an index TCC might take.

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Loss of heterozygosity (LOH), at the region of a known tumour suppressor gene, is considered indicative of a mutation in the remaining copy of the gene and loss of the tumour suppressing function of the gene product. LOH analysis of sequential carcinomas from TCC patients would, therefore enable tracking of tumour suppressor gene inactivation in relation to recurrence (Bartlett et al, 1998; Wagner et al, 1999; Watters et al, 2000).

Deletions involving all or part of chromosome 9 are the most frequently reported genetic event in TCC of the urinary bladder (Bartlett et al, 1998; Knowles, 1998, 1999; Czerniak et al, 1999). We have recently demonstrated that deletion of *TSC1* on chromosome 9 predicts recurrence in a subset of TCC patients (Edwards et al, 2000). However, in order to identify further recurrence-related genes, other areas of deletion associated with bladder cancer should be investigated. The current study has selected areas of deletion previously associated with TCC of the bladder. LOH at 4p16.3 has been reported in over 1/3 of bladder cancer cell lines (Bell et al, 1996) and deletions in 8p are associated with high-grade carcinomas and distant metastasis of urinary bladder cancers (Ohgaki et al, 1999). LOH on both 11p and 11 q have frequently been reported in TCC of the bladder (Fearon et al, 1985; Shaw and Knowles, 1995; Voorter et al, 1996; Monaco et al, 1997). Loss of 17p13.1, the *TP53* locus, is associated with high tumour grade (Tsutsumi et al, 1997; Esrig et al, 1993; Chaturvedi et al, 1997) and invasive growth in bladder cancer (Tsutsumi et al, 1997). Loss of 17q22 and 17q24-25 have also been reported (Chaturvedi et al, 1997).

This study investigated LOH in 109 TCCs of the urinary bladder from 46 patients with known follow-up. The patients fell into two groups: those with carcinomas that did not recur and those with carcinomas that recurred and/or progressed. LOH was investigated using a panel of microsatellite markers that spanned chromosomes 4, 8, 11 and 17.

MATERIALS AND METHODS

Patients and samples

Forty-six patients were selected for this retrospective study. Ethical approval was gained from the local ethics board. Patients either had non-recurrent carcinomas (NR; $n = 17$) and were disease free with follow-up for a minimum of 3 years or had recurrent carcinomas (REC; $n = 29$). These patients had several episodes of recurrent TCC after the index carcinoma.

Archival (formalin fixed, paraffin embedded) TCCs were regraded and staged by KMG using UICC criteria (UICC, 1978). Tissue sections dewaxed in xylene (2 × 10 min), and rehydrated through 99% alcohol (2 × 2 min) and 95% alcohol (2 min) were stained in 0.05% Toludine blue for 30 s. Areas of tumour were microdissected from 5 µm sections. DNA was extracted by incubating for 4–7 days at 37˚C in 25 µl of proteinase K in digestion buffer (0.5 mg/ml proteinase K, 0.5% Tween 20 in 50 mM Tris-EDTA pH 8.5) (Going and Lamb, 1996). The proteinase K was inactivated by heating at 95˚C for 10 min. Normal tissue (e.g. detrusor muscle) was also dissected from the same section. LOH analysis was conducted using 1 µl aliquots of DNA without further purification.

PCR

Twenty-six microsatellites spanning four chromosomes were investigated (Table 1). Primers were from MWG-Biotech UK Ltd or Perkin-Elmer Applied Biosystems, with one primer from each pair fluorescently labelled at the 5′ end all primer sequences are available from the authors on request.

Target sequences were amplified by PCR in 10 µl reactions (1× reaction buffer containing 10 pmol of each primer, 3 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP and 0.5 units hot start Taq polymerase (Qiagen Ltd, Sussex, UK)). The reaction was started after 15 min denaturation of DNA at 95˚C. DNA amplification was performed in a MJ Research PTC – 225 Peltier Thermal Cycler (Genetic Research Instrumentation, Essex, UK) as follows: 45 cycles of denaturation at 94˚C for 30 s, annealing at 47, 49, 50, 51 or 53˚C (Table 1) for 30 s and extension at 72˚C for 1 min, followed by a final extension for 15 min at 72˚C.

PCR analysis

Genotyping was performed on an automated laser activated fluorescent DNA sequencer (Perkin Elmer ABI 377 sequencer) by DNASHEF Technologies, Edinburgh. Fluorescent gel data were collected automatically during electrophoresis and analysed using Genescan™ software, peak size, height and area were measured. Allele loss was assessed as described by Niederacher et al, (1997), i.e. the allele ratio of carcinoma DNA peak height was divided by the allele ratio of paired normal DNA peak height. A ratio below 0.65 or above 1.55 was considered indicative of loss. Repeat analysis was performed on random samples to confirm reproducibility.

Table 1 Outlines the microsatellites studied, their chromosomal region and the annealing temperature of their PCRs

Microsatellite markers	Region	Temperature (˚C)
D4S174	4p11–15	50
D4S127	4p16.3	53
D8S254	8p22	50
D8S133	8p22	50
D10S215	10q23.1	47
D11S922	11p15.5	53
D11S569	11p15.1	50
FGF3	11q13	53
D11S490	11q23.3	50
D17S578	17p13.3	49
D17S849	17p13.3	50
D17S960	17p13.1	49
TP53	17p13.1	50
D17S786	17p13.1	49
D17S1852	17p13.1	50
D17S799	17p12	50
D17S921	17p12	50
D17S1857	17p12	50
D17S798	17q11–12	50
D17S932	17q21	49
D17S579	17q21	51
D17S943	17q21	49
D17S944	17q21	51
D17S807	17q21	49
D17S949	17q21	50
D17S784	17q24–25	51
D17S928	17q24–25	51

Statistics

Differences between patient groups were analysed using Fisher's exact test. Disease free survival was evaluated using the Kaplan-Meier method and curves were compared with the log rank test.

RESULTS

There were no significant differences between patient age or sex or index carcinoma stage or grade when the NR and REC groups were compared. The stage and grade of all carcinomas used were known (Table 2). The success rate of LOH analysis from archival carcinoma material was greater than 95%.

Index carcinomas

The frequency of LOH at all informative markers spanning chromosomes 4, 8, 11 and 17 were compared in index NR and index REC

Table 2 Shows the stage and grade of carcinomas, NR denotes non recurrers, index (REC) denotes the index carcinomas of patients with subsequent recurrences and recurrent (REC) denotes the recurrent carcinomas

	Stage				Grade		
	Ta	T1	T2	Other	1	2	3
NR	13/17	4/17			6/17	11/17	
Index (REC)	17/29	9/29		3/29	11/29	10/29	5/29
Recurrent (REC)	37/63	11/63	15/63		21/63	18/63	24/63

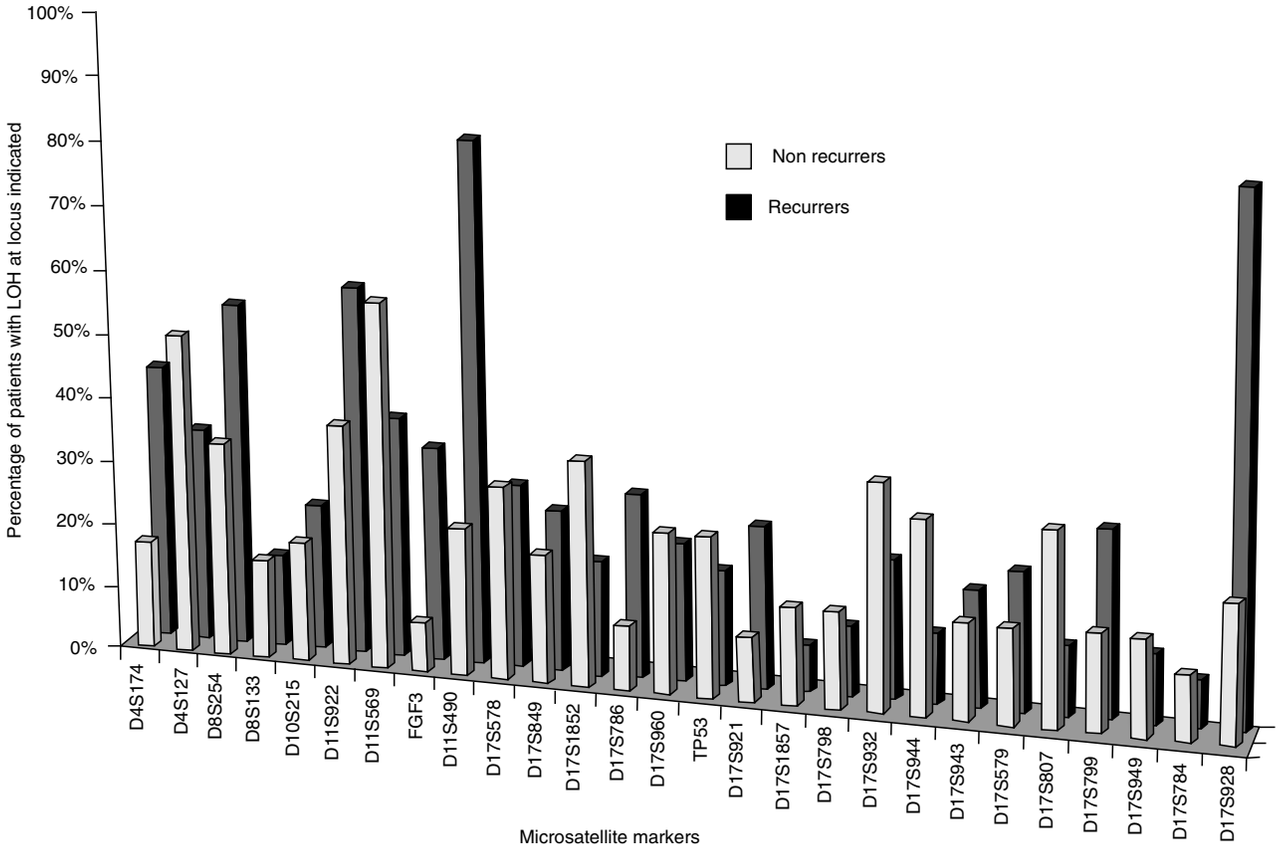

Figure 1 LOH frequency at each loci for the index carcinomas of the NR and REC groups

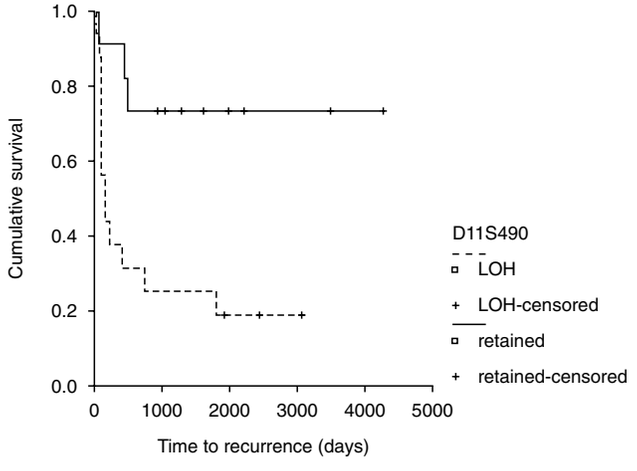

Figure 2 Disease free survival of 28 patients who retained or had LOH at D11S490 ($P = 0.0079$)

carcinomas; no significant differences were identified (Table 3). No significant differences were identified when loss of chromosomal regions were compared for index NR and index REC carcinomas.

The frequency of loss of D11S490 was significantly higher in patients who recurred compared to patients who did not recur (NR, 25%, 3/12 versus REC, 81%, 13/16, $P = 0.004$) (Figure 1). This held true when pTa carcinomas alone were considered (NR, 20% 2/10, versus REC 89%, 8/9, $P = 0.001$). Time to recurrence was significantly longer in patients who retained D11S490 than in those who lost it (Figure 2, $P = 0.0079$); 16 patients out of 28 informative cases had LOH at D11S490.

Table 3 Shows the frequency of LOH at all informative markers spanning chromosome 4.8, 11 and 17 in two patient groups. NR = non-recurrers and REC = recurrers

	NR	REC
Chromosome 4	29% (5/17)	21% (6/28)
Chromosome 8	12% (2/17)	3% (1/28)
Chromosome 11	0% (0/17)	18% (5/28)
Chromosome 17	0% (0/17)	0% (0/28)

The frequency of loss of D17S928 was significantly higher in the index carcinomas from the REC group compared to NR carcinomas (NR, 21%, 3/14 versus REC, 80%, 16/20, $P = 0.002$) (Figure 1). This held true when the pTa carcinomas alone were considered (NR, 15%, 2/13 versus REC, 83%, 10/12, $P = 0.002$). Time to recurrence was significantly longer in patients who retained D17S928 than in those who lost it (Figure 3, $P = 0.04$); 19 patients out of 34 informative cases had LOH at D17S928.

LOH data from D11S490 and D17S928 identified 23 out of the 29 patients as likely to recur compared to 13 for D11S490 and 16 for D17S928. All patients were included and none were lost due to non informative markers. Time to recurrence was significantly shorter in patients who had LOH at either D11S490 and D17S 928 than in those who had retention at either or both ($P = 0.03$).

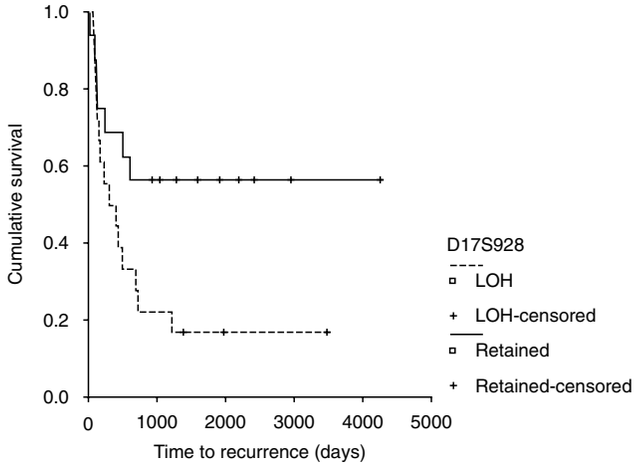

Figure 3 Disease free survival of 34 patients who retained or had LOH at D17S928 ($P = 0.04$)

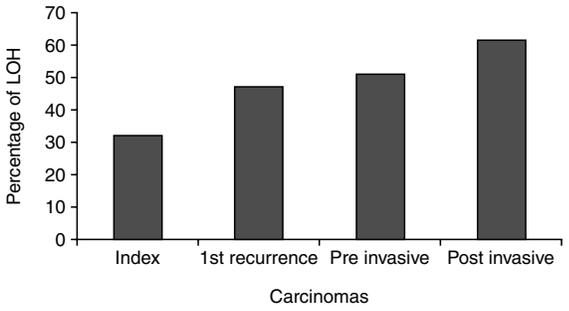

Figure 4 Overall percentage of LOH noted in index, first recurrence, immediately pre-invasive and muscle invasive carcinomas of patients who presented with a low grade carcinoma which recurred and progressed to a muscle invasive carcinoma (RP)

The trend in total LOH frequency as a carcinoma recurs and progresses

The trend of LOH in recurrent carcinomas was investigated, the REC group being sub-divided into carcinomas that recurred (RNP) and carcinomas that recurred and progressed to higher stage disease (RP). The frequency of LOH in each group was calculated by dividing the total number of informative cases by the total number of LOHs in each group and multiplying by 100, therefore the frequency was expressed as a percentage. In the RNP group there was no significant difference in the frequency of LOH observed in index and recurrent carcinomas (index tumour 40% (116/316), first recurrence 47% (146/310) and last recurrence 48% (117/241). In the RP patient group the frequency of LOH increased with progression: 32% (66/205) of index carcinomas, 47% (88/ 187) of first recurrent carcinomas, 51% (99/192) of immediately pre-invasive carcinomas and 61% (90/148) of post-invasive carcinomas. As the index carcinoma progressed to an invasive carcinoma a significant increase in the frequency of LOH was noted ($P > 0.0001$) (Figure 4).

The significance of LOH levels in carcinomas grouped by grade and stage

When carcinomas were grouped by stage and the frequency of LOH compared for individual loci or chromosomal regions the only significant difference was at 8p22, pTa carcinomas had a significantly lower frequency of LOH than pT1 carcinomas (19/39, 48% versus 10/11, 91% $P = 0.024$).

When the frequency of LOH was compared for carcinomas grouped by grade, loss of all informative markers spanning the *TOP II* region on chromosome 17 was found to increase with grade. (Grade 1, 2/33, 6% versus grade 2, 9/25, 36%, $P = 0.01$; and grade 1, 2/33, 6% versus grade 2 and 3, 15/49, 25%, $P = 0.01$).

DISCUSSION

Current molecular and pathological parameters fail to predict which early TCCs will recur and/or progress. This study assessed genetic changes on chromosome 4, 8, 11 and 17 in two patient groups in an attempt to identify genetic changes in index carcinomas that would predict the path a carcinoma might follow. The index carcinomas investigated were either pTa or pT1. The results were analysed with pTa and pT1 index carcinomas combined and separated as genetic differences between pTa and pT1 carcinomas have now been identified (Richter et al, 1997; Simon et al, 1998). Interestingly only one marker had significantly different frequency of LOH when the carcinomas were grouped by stage (8p22 between pTa and pT1) or grade (17p21 between grade 1 and grade 2). These results suggest that the frequency of LOH at the microsatellite loci used in this study is independent of stage or grade.

Frequent deletions on both arms of chromosome 11 in TCC of the urinary bladder indicate the probable location of relevant tumour suppressor gene/s (Fearon et al, 1985; Voorter et al, 1996). Distinct regions of deletion exist on 11p (11p13 and 11p15) and 11q (11q13–q23.3), and LOH in these regions is associated with high grade carcinomas (Shaw and Knowles, 1995). Chromosome 11 analysis in this study focused on these regions. The frequency of LOH was compared at 11p13, 11p15, 11q13 and 11q23 in index carcinomas from NR and REC patients. There was more frequent LOH at D11S490 (11q23) in index REC carcinomas than NR carcinomas ($P = 0.004$). Chromosomal deletions at region 11q23, have been associated with human carcinomas including lung (Iizuka et al, 1995), ovarian (Foulkes et al, 1993) and uterine cervix (Hampton et al, 1994), suggesting the presence of at least one tumour suppressor gene in this area. Several candidate genes linked to carcinogenesis at 11q23 include *Cbl*, *EST1*, *DDX6*, tumour suppressor gene on chromosome 11 (*TSG11*) (locus link) and *LOH11CRA* (Monaco et al, 1997). The two most likely candidate genes are *Cbl* and *TSG11*. Cbl protein is a central player in the regulation of tyrosine kinase signalling pathways and is therefore implicated in many growth and apoptotic pathways (Lupher et al, 1998). *TSG11* has been demonstrated as a tumour suppressor gene in lung cancer and possibly a variety of other cancers (Murakami et al, 1998). Although *LOH11CRA* is hypothesized as a possible carcinoma suppressor gene its function is unknown. Further investigations are required but it appears that deletion of 11q23 represents an important genetic event in the recurrence of bladder cancer.

In TCC of the bladder LOH studies on chromosome 17 have tended to focus on 17p13 where *TP53* lies, there is therefore relatively little information available on loss of 17q. Losses at 17q11–22 and 17q24–25 have, however, been reported (Chaturvedi et al, 1997). The current study therefore investigated LOH on chromosome 17 using 18 microsatellites that span the chromosome, 8 of these markers are on 17q. The frequency of LOH at individual markers spanning chromosome 17 ranged from 7% to 80%

depending on the locus investigated. D17S928 (17q25) had the highest frequency of LOH, and was associated with recurrence. Loss of 17q25 is found in breast and ovarian carcinomas (Kalikin et al, 1996, 1997) and is independent of *BRCA 1* deletions. LOH and functional suppression studies in breast, ovarian and oesophageal cancer, support the existence of a novel tumour suppressor gene at 17q24–25 (Jacobs et al, 1993; Godwin et al, 1994; Theile et al, 1995; Plummer et al, 1996; Petty et al, 1998). Candidate genes at 17q24–25 include Thymidine kinase 1 (*TK1*) and apoptosis inhibitor 4 (*API4*). *TK1* encodes soluble thymidine kinase (TK1) which catalyses the phosphorylation of thymidine to deoxythymidine monophosphate and is associated with cell division (Petty et al, 1998). LOH at 17q24–25 is postulated to increase TK1 activity in breast cancer (Petty et al, 1996), and TK1 levels in breast tumours and serum predict recurrence (O'Neill et al, 1992). *AP14* encodes for survivin, which has abundant expression in lung, pancreas, breast and prostate cancer (Ambrosini et al, 1997). Survivin is expressed in G2/M and associates with microtubules of the mitotic spindle at the beginning of mitosis (Li et al, 1998). It is believed to counteract induction of apoptosis in G2/M (Li et al, 1998). Therefore, LOH at this region might interfere with the cell cycle and promote recurrence of TCC of the urinary bladder. Further mapping studies of this area of chromosome 17 and functional studies are required to confirm a role for these candidate genes.

The total frequency of LOH increased with recurrence and progression, demonstrating an accumulation of genetic defects throughout the disease history, but whether this is a cause or a consequence of TCC progression is unknown. Increased LOH with progression was most noticeable at TP53, D17S807, D11S922 and D11S569 (data not shown). Increased LOH at TP53 with carcinoma progression is not surprising as *TP53* alterations are frequently associated with muscle invasivion (Spruck et al, 1994; Sidransky et al, 1991; Chaturvedi et al, 1997).

Genetic differences exist between low grade TCC which may determine subsequent tumour behaviour and identification of the associated tumour suppressor genes is required for better understanding of bladder carcinogenesis.

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