High-Resolution Deletion Mapping of 15q13.2-q21.1 in Transitional Cell Carcinoma of the Bladder

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ABSTRACT

Deletions found in several types of human tumor, including carcinomas of the colorectum, breast, and lung, suggest the presence of a potential tumor suppressor gene(s) on chromosome 15. Common regions of deletion in these tumors are at 15q15 and 15q21. Here, we have analyzed loss of heterozygosity (LOH) on chromosome 15 to ascertain its potential involvement in the development and progression of transitional cell carcinoma (TCC) of the bladder. A panel of 26 polymorphic markers, spanning 15q12–15q22, were used to map regions of LOH in 51 TCCs. LOH was found for at least one marker in the region 15q14–15q15.3 in 20 of 51 (39%) tumors. Deletion mapping defined two minimum regions of deletion: a distal region between the markers D15S514 and D15S537 at 15q15.1–15q15.3 (estimated as 3 Mb) and a more proximal region between the markers D15S971 and D15S1042 at 15q14 (estimated as 1.1 Mb). Analysis of a panel of 33 bladder tumor cell lines revealed regions of contiguous homozygosity for markers in 15q15, indicating likely LOH. Fluorescence in situ hybridization analysis demonstrated that mitotic recombination is the predicted mechanism of LOH in two of these. These regions of LOH on 15q may contain tumor suppressor genes the loss or inactivation of which is associated with TCC development. The DNA repair gene RAD51 at 15q15.1 represents a candidate 15q tumor suppressor gene. Expression analysis of rad51 protein in tumor cell lines revealed variable levels of expression but no significant loss of expression in cell lines with likely 15q LOH.

INTRODUCTION

Bladder cancer is one of the most common solid epithelial cancers, representing the fourth most common cancer in men in the United Kingdom and United States. The majority of bladder tumors are TCCs,1 and these fall into two major groups with distinct clinical features. More than 80% are superficial papillary tumors, only a small proportion of which (10–15%) progress to invade muscle. Although such tumors are relatively nonaggressive, they recur frequently (70%) and require long-term cystoscopic surveillance with associated morbidity and high cost. The remaining 20% of tumors present as invasive lesions with poor prognosis. Five-year survival for this latter group is ~50%. The distinct clinical behavior of these two groups of TCCs indicates likely genetic differences that might provide useful markers for diagnosis, disease monitoring, and prognosis.

Genetic and cytogenetic analyses have identified many alterations in TCC, the majority of which are found predominantly in tumors of high grade and stage (1, 2). These include inactivating mutations of TP53 and RB1, amplification of ERBB2 and a large number of nonrandom genomic deletions, and amplifications identified by LOH and CGH analyses. The latter include LOH of chromosome arms 3p, 4p, 4q, 5p, 5q, 8p, 10q, 11p, 14q, and 18q and amplifications on 1q, 3p, 6p, 8q, 10p, 10q, 11q, 12q, 17q, and 20q. In contrast, in papillary superficial tumors, few alterations have been found at high frequency, with the exception of deletions of chromosome 9 found in ~50% of cases (3) and mutation of the fibroblast growth factor receptor 3 gene (FGFR3) found in 70% (4). Because bladder cancer is a disease of middle and old age, it is predicted that additional heritable alterations contribute to the development of superficial bladder tumors, and these remain to be identified.

We have previously performed an allelotype analysis of TCC (5), and there have been several comprehensive CGH and cytogenetic analyses of both superficial and advanced bladder tumors that have revealed no novel frequent alterations in superficial tumors. If additional genetic events are present, these may be predominantly small alterations not detectable at the resolution of these genomic screening strategies. Nevertheless, the likely location of these might be indicated by some of the less frequent gross genomic alterations reported. Monosomy 15 detected by FISH on bladder irrigation specimens has been reported in diploid TCC and some hyperdiploid tumors (6), but CGH analyses have not found a significant frequency or either under- or over-representation of chromosome 15 (7–9). To date, no detailed examination of chromosome 15 by LOH has been performed in TCC. Studies of breast, colorectal, parathyroid, ovarian, and lung carcinoma and malignant mesothelioma have indicated several potential tumor suppressor loci on chromosome arm 15q. LOH studies have shown deletions at 15q11–q13 (10), 15q14 (11), 15q15 (12–14), 15q21–22 (10, 15, 16), and 15q26 (10, 17). Evidence has also been found for a predisposition gene for colorectal adenomas and carcinomas at 15q14-q22 (18). Additional evidence for the presence of a tumor suppressor gene on 15q has been provided from in vitro studies by Boukamp et al. (19), who reported loss of 15q at a late stage in malignant conversion in a cell-culture system using HRAS transfected skin keratinocytes.

A number of genes mapped to the common regions of 15q LOH have been considered as candidate tumor suppressor genes, including the DNA repair gene RAD51, thrombospondin (THBS1), and the transforming growth factor β family genes SMAD3 and SMAD6. Mutation analyses of RAD51, SMAD3, and SMAD6 have found no somatic mutations in tumors with 15q LOH (16, 20, 21).

In this study, we have analyzed the incidence of LOH on chromosome 15q in 51 bladder tumors of various grades and stages, to ascertain its potential involvement in the development and progression of TCC. We have also performed microsatellite typing of bladder tumor-derived cell lines to determine their likely LOH status and metaphase FISH to allow deletion of small interstitial regions of 15q to be distinguished from mitotic recombination events. Our findings indicate that reduction to homozygosity of markers on 15q is common in TCC and that, in at least some cases, this is accomplished by mitotic recombination. Two small regions of interstitial deletion have been mapped within which candidate gene identification can now begin.

MATERIALS AND METHODS

Patients and Tissues. Bladder tumors were obtained by surgical resection from St. James’s University Hospital, Leeds. All patients gave informed
Some markers were amplified together, using varying primer concentrations. Informative results were repeated at least twice in single-marker reactions. Allele products were analyzed by Genescan software (Applied Biosystems). Each and electrophoresed in 4.25% polyacrylamide gels containing 7 M urea, and the following cycle parameters: 95°C for 30 s, 55°C for 30 s, followed by a final extension of 72°C for 30 s, 72°C for 10 min, then 29 cycles of 95°C for 10 min and a final incubation of 60°C for 30 min. Each PCR reaction was using AmpliTaq Gold (Applied Biosystems, Warrington, United Kingdom) sequences from tumor DNA and corresponding normal DNA were amplified using AmpliTaq Gold (Applied Biosystems, Warrington, United Kingdom) and the following cycle parameters: 95°C for 10 min, then 29 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by a final extension of 72°C for 10 min and a final incubation of 60°C for 30 min. Each PCR reaction was performed under standard conditions in a 12.5-μl reaction volume containing 1 μl of template DNA (−10 ng), 0.8 μM of each primer, 0.2 μM of each dNTP, 1.5 mM MgCl2, 0.2 units of Taq polymerase, and 1.25 μl of 10× PCR buffer. Some markers were amplified together, using varying primer concentrations and 2–3 μl of template DNA. Reaction products (1 μl) were then denatured and electrophoresed in 4.25% polyacrylamide gels containing 7 M urea, and the products were analyzed by Genescan software (Applied Biosystems). Each informative result was repeated at least twice in single-marker reactions. Allele ratios for tumor compared with normal DNA were calculated as (A1/A2)/T/(A1/A2)/N. LOH was scored if this ratio was <0.4.

**Western Blot Analysis.** Tumor cell lines were analyzed for expression of rad51 protein. Cells were lysed during logarithmic growth phase in 60 mm Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and protease inhibitor mixture (Sigma). DTT and bromphenol blue (100 mM and 0.025%, respectively) were added, and lysates were boiled for 5 min. Protein lysates were resolved by electrophoresis (20 μg/track) in 15% polyacrylamide gels and transferred to nitrocellulose membranes (Hybond C; Amersham Biosciences). The filters were blocked with 5% nonfat dried milk and incubated overnight with mouse anti-rad51 antiserum at 1:1000 (Abcam ab213-100), incubated with horseradish peroxidase-conjugated antinnos IgG (Southern Biotechnology Associates, Birmingham, AL), and washed. Antibody binding was visualized by chemiluminescence (Amersham Biosciences). The blots were then washed, incubated with mouse anti-β-actin antibody diluted 1:10,000 (Sigma clone AC-15), and detected as above. The intensity of the rad51 signals was normalized to that of β-actin with ImageQuant software (Molecular Dynamics).

**FISH Analysis.** Metaphase chromosomes were prepared according to standard procedures. BACs (RP11-6403 containing CXA9, RP11-532F12 containing RAD51, RP11-27P20 containing D15S537, and RP11-27M9 containing THBS1) were obtained from the Sanger Institute (Hinxton, United Kingdom) and labeled with biotin by a nick translation kit (Intergen). One hundred sixty

![Fig. 1. Pattern of LOH in bladder tumors with deletions of 15q. Microsatellite markers and their positions in megabases from 15pter according to Ensembl (release 7.299.2; July 12, July 2002) are shown on the left. Two common regions of deletion are denoted by vertical bars 1 and 2. Five tumors on the right show deletions in regions 1 and 2 but each also contain deletions of at least one marker that is not compatible with regions 1 and 2. These identify an additional three possible regions denoted by *, †, and ‡.](image-url)
RESULTS

Definition of Two Common Regions of Deletion on Chromosome 15q. LOH analysis was performed on 51 TCC samples using 26 highly informative microsatellite markers spanning 15q13.2–15q21.1. All patients were informative for multiple markers, allowing detailed deletion maps to be constructed for their tumors. LOH was found in the region 15q14-q15.3 in 20 of 51 (39%) tumors (Fig. 1). Eleven tumors showed LOH in a single overlapping region at 15q15.1. Assuming that all of these deletions target the same locus, this defines a common region between 15q14-q15.3 in 20 of 51 (39%) tumors (Fig. 1). Eleven tumors showed LOH in a single overlapping region at 15q15.1. Assuming that all of these deletions target the same locus, this defines a common region between 15q14-q15.3 in 20 of 51 (39%) tumors (Fig. 1). Examples of electropherograms illustrating the flanking markers of this region are shown in Fig. 2. This region was also deleted in three other tumors (312, 467, and 323) that had deletion in other regions of 15q.

Four tumors showed LOH in a single region at 15q14 with a single region of overlap between D15S514 and D15S537, estimated as 3 Mb and defined by proximal breakpoints in tumors 438, 499, 309, and 387 and by distal breakpoints in tumors 372, 309 and 458 (region 1; Fig. 1). Examples of electropherograms illustrating the flanking markers of this region are shown in Fig. 2. This region was also deleted in three other tumors (312, 467, and 323) that had deletion in other regions of 15q.

Information on tumor grade was available for 48 of the tumors analyzed and on stage for 46 tumors. No significant association was found between chromosome 15q LOH and tumor grade and/or stage. Assessment of 15q Deletion Status in Bladder Tumor Cell Lines. A panel of 33 bladder tumor-derived cell lines was examined for possible LOH on 15q using 13 markers mapped to 15q14-q15 and encompassing deletion regions 1 and 2 defined in tumors. Paired normal DNA was available for only one of the lines, which showed no LOH. Therefore, we used frequencies of homozygosity at highly polymorphic loci to predict likely LOH. Fig. 3 shows allele counts in the cell lines. In three cell lines, likely LOH across the entire region was shown by the presence of only a single allele at all loci. Four other cell lines showed single alleles at all but one locus analyzed, also indicating likely LOH with possible genetic rearrangement, resulting in retention of both alleles for only a small fragment of the region. Contiguous homozygosity for three or more markers was also considered to indicate possible LOH and was found in an additional eight cell lines. Calculated frequencies of heterozygosity were compared with expected frequencies (Genome Database) and were significantly lower at several loci. The greatest difference was found at D15S194 and D15S1012 (36% and 56% of expected frequencies, respectively).

Assessment of DNA Copy Number Changes in Regions of LOH by FISH. Because the regions of LOH mapped in the tumor panel were small and interstitial and many were found in low-grade and low-stage tumors that are often karyotypically near-diploid, we considered the possibility that these events may be generated via mitotic recombination or gene conversion events rather than deletion. If this is the case, no copy number change is expected within the region of LOH. We assessed this by FISH analysis with four BAC clones selected to contain genes and/or markers within the critical regions of LOH. These were the genes connexin 9 (CXA9), which maps close to D15S118 in region 2; thrombospondin 1 (THBS1), which maps proximal to region 1; RAD51, which is within region 1; and D15S537, which lies distal to region 1 (Fig. 4). Because no fresh tumor tissues were available from tumors with critical deletions, we used two cell lines with a high probability of LOH in region 2. JO’N (Fig. 3, cell line 7) is homozygous for markers spanning the region that contains CXA9 and THBS1 but has two alleles both proximal and distal to RAD51 and is heterozygous for D15S537. FISH analysis with the four probes and a chromosome 15 centromeric probe showed that JO’N had two copies of chromosome 15, both of which retained all four probe sequences. Results for CXA9 and D15S537 are shown in Fig. 4A. SW1710 (Fig. 3, cell line 10) also has a region of homozygosity extending across region 2, including CXA9 but not the other three probes. Again, signals for all four probes were detected on each of the three or four copies of chromosome 15 in SW1710 (Fig. 4B). This is compatible with reduction to homozygosity via double recombination between homologous chromosomes.

Rad51 Expression in Bladder Tumor Cell Lines. Western blot analysis was performed to examine expression of rad51 protein in a panel of cell lines with and without predicted 15q LOH and in two cell lines with finite in vitro life spans derived from normal human urothelium from different donors. Expression levels relative to a β-actin control are shown in Fig. 5. Levels of expression did not show an obvious correlation with predicted LOH status, and although levels did vary between cell lines, in no case was expression markedly lower at several loci. The greatest difference was found at D15S194 and D15S1012 (36% and 56% of expected frequencies, respectively).
reduced or absent. Interestingly, one of the two normal cell lines analyzed (NHU122) showed the lowest level of rad51 protein.

**DISCUSSION**

We have identified two common regions of deletion on chromosome 15 in transitional cell carcinoma of the bladder, a proximal minimal region between the microsatellite markers D15S971 and D15S1042, and a second, more frequently deleted distal region between D15S514 and D15S537. Apart from one deletion, all regions of LOH identified were small interstitial regions and would have been missed by all previous allelotype and CGH studies of TCC. This is in contrast to findings in previous studies, including those of colorectal carcinoma (15), lung carcinoma (10), and mesothelioma (14), in which large, frequently terminal deletions of 15q were common. Differences in the pattern of LOH may reflect different mechanisms for the generation of homozygosity in different tumor types or may indicate the presence of several target genes that are relevant to some other tumor types. Nevertheless, the two regions we have defined are contained within the larger regions mapped by others, and our findings may localize tumor suppressor loci relevant to several other major tumors.

The most comprehensive study of allelic loss on 15q, to date, was in malignant mesothelioma (14). This study used 26 markers spanning 15q11-q26. LOH was identified in 48% of cases and defined a single minimum region of deletion of ∼3 cM at 15q15, which was confirmed by FISH analysis. The flanking markers of this region were D15S1007 and ACTC, which places the critical region proximal to and nonoverlapping with our region 2 (defined in tumor 304). A single tumor in the study by De Rienzo et al. (14) did show an interstitial deletion distal to D15S118, which is coincident with our region 2. Many of the tumors in this latter study had large terminal deletions encompassing both of the regions mapped here and possibly involving loss of function of the same gene(s).

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**Fig. 3.** Allele counts for markers at 15q14-q15 in 33 bladder tumor cell lines. Regions of homozygosity for three or more markers are gray. Two markers within critical regions 1 and 2 defined in tumors are gray. Each allele is denoted by ●. Observed heterozygosity frequencies were compared with expected heterozygosities obtained from the Genome Database. Figures on right note observed heterozygosity as a percentage of expected.

**Fig. 4.** FISH showing retention of chromosome 15q copy number in regions of predicted LOH in bladder tumor cell lines. Vertical bars indicate allele counts for 15q markers and positions of FISH probes containing CXA9, THBS1, and D15S537. a, cell line JO’N, which has predicted LOH of CXA9, showing retention of CXA9 (top, green) and D15S537 (bottom, green) on both copies of chromosome 15 (centromeric probe, red); b, cell line SW1710 with predicted LOH encompassing CXA9, showing retention of CXA9 signals (top, green) and THBS1 (bottom, green) on all copies of chromosome 15 (centromeric probe, red). SW1710 contains clones with three or four copies of chromosome 15.
A study of brain metastases from a range of carcinomas, mostly breast, also mapped a region of deletion on 15q, between the markers GAAA1C11(D15S1232) and D15S641 (11). This overlaps with our region 1. Similarly, regions mapped in colorectal carcinoma (15) and in pancreatic carcinoma (23) contain our region 1. If these deletions all target the same gene, our present findings significantly narrow the region within which a search for candidate genes should now focus.

Region 2 is very small and is estimated as ~1 Mb. A previous linkage and LOH analysis in an Ashkenazi family with dominant inherited predisposition to colorectal adenomas and carcinomas found evidence for linkage to 15q14-q22, with a maximum LOD score at D15S118 (18). This marker was the most frequently deleted marker in bladder tumors in region 2. This region contains only a few genes, which can now be assessed as candidate genes.

Region 1 is estimated as 3 Mb and contains 16 known genes, including p53-binding protein 1, TP53BP1, and a D-type cyclin-interacting protein, CCNDBP1. Interestingly, a recent publication has identified a feline orthologue of one of the genes in this region (FLJ12973/Q9H967) as a candidate tumor suppressor gene potentially inactivated by proviral insertion in a feline lymphoma (24). This gene, therefore, represents a candidate worthy of further analysis. Currently, however, the region remains too large for systematic mutation analysis of candidate genes, but additional screening of new tumors using a reduced marker set spanning only region 1 should allow the rapid identification of additional tumors with small deletions to refine this localization.

Previous studies identified RAD51 as a possible candidate tumor suppressor gene on 15q15. This gene is a structural and functional homologue of the E. coli RecA recombinase and plays a key role in the repair of double-strand breaks by homologous recombination. The gene is contained within several of the larger deletions found here and many deletions described in other studies. However, during the course of our study, a new build of the genome map just excluded RAD51 from deletion region 1. Previous studies have failed to detect mutations in the retained allele of RAD51 in tumors with 15q LOH (21, 25). However, this is perhaps not surprising given the lethality of targeted disruption of the gene in the mouse germ line or in embryonic stem cells (26–28). We considered it possible that loss of one copy of the gene might cause sufficient down-regulation of expression of rad51 to generate a DNA repair-deficient phenotype. However, we found no evidence in cultured bladder tumor cells that likely 15q15 LOH was associated with a reduction in expression levels of rad51. Indeed, quite variable levels of expression were measured, including a very low level of expression in one of the normal urothelial cell cultures we assessed. A role for RAD51 cannot be excluded based on these findings, but it is unlikely that RAD51 represents a tumor suppressor gene inactivated by the classical two-hit mechanism. Elevated levels of expression of rad51 have been reported in a range of tumor cells in vitro, although no bladder tumor cells were assessed (29). At present, we do not know whether the levels of rad51 protein detected in bladder tumor cells was abnormally high, because the level in the two normal urothelial cell lines used varied considerably. It will be of interest to assess both overall protein levels and the presence of rad51 nuclear foci in these cells.

No common fragile sites have been described in this region, but a site induced by camptothecin on 15q15 has been described in a single publication (30). To date, no information about the precise localization on the genomic sequence has been obtained.

A surprising finding in this study was increased allelic instability in several of the tumors studied. Generally, this was found in those tumors that showed several regions of LOH on 15q (Fig. 1, tumors 484, 355, 467, and 323). Widespread microsatellite instability at dinucleotide repeats is not common in bladder cancer (31, 32). In contrast, elevated microsatellite instability at tetranucleotide repeats has been described at high frequency (43.9%) in bladder cancer and seems to be associated with TP53 mutation (33). It has been suggested that p53 mutant tumor cells may have increased tolerance to this type of defect. The reason for the observed dinucleotide repeat instability in the present study is not clear but could reflect a particular feature of the genome in this region of 15q or may reflect a specific replication or repair defect in these tumors that might lead to an increased rate of LOH.

In other tumor types, 15q LOH has been described as a relatively late event. In ovarian cancer, it is observed mostly in high-grade tumors and has been proposed as a late event (34); in breast carcinoma, LOH was more frequent in metastatic than in primary tumors (11). In this series of bladder tumors, which included tumors of all grades and stages, we found no evidence for an association of 15q LOH with either grade or stage. This is similar to the situation for chromosome 9 LOH and may indicate that a gene or genes on 15q contributes early in the process of bladder tumor development.

We have identified several cell lines with likely LOH of 15q, and the regions of highest homozygosity indicate likely involvement of the same critical regions mapped in tumors. Interestingly, in contrast to our findings in tumors, several cell lines seemed to have LOH involving the entire region analyzed. These lines will be useful for future mutation and functional studies of candidate genes.

LOH is a very common finding in cancer cells, but, to date, few studies have attempted to elucidate the underlying mechanisms of LOH at the DNA level. However, it has been shown that mitotic recombination or gene conversion, and not deletion, is responsible for some LOH events. For example, in neurofibromas from NF1 patients, inactivation of the second NF1 allele was shown to be commonly via interstitial 17q LOH with no reduction in 17q copy number (35). There is also the suggestion that the mechanism of LOH may be chromosome specific (36). To date, the only information on chromosome 15 has come from the study by De Rienzo et al. (14), in which two mesothelioma samples with 15q LOH were shown by FISH to have copy number loss within the region of interest. Our data indicate that LOH for small regions of 15q is not accompanied by physical deletion of a DNA copy of the region. This suggests that double mitotic recombination is likely to be the mechanism by which at least some interstitial LOH on 15q is accomplished in bladder tumors. It is highly probable from our finding of multiple contiguous homozygous microsatellite loci that the two cell lines studied have 15q LOH. Nevertheless, the lack of availability of paired nontumor cells leaves open the possibility that these lines came from individuals with germ-line homozygosity of multiple adjacent markers. It will now be important to confirm this finding in fresh tissue samples from tumors in which paired normal tissue is available. Our finding of several cell lines with more extensive homozygosity for 15q markers than the tumors studied may indicate in vitro selection of cells with more extensive LOH or may indicate that genetic evolution has occurred in culture. These questions also require paired normal DNA or cultured...
cells for each tumor cell line, providing a strong impetus for the establishment of novel bladder tumor cell lines with paired lymphoblastoid controls that will allow maximum exploitation of available molecular genetic analyses.

In conclusion, we have mapped two small interstitial regions of LOH on 15q in bladder cancer. Many other types of tumor have frequent deletion of proximal 15q, including breast (11), colorectal (15), mesothelioma (14), pancreatic (23, 37), head and neck squamous cell carcinoma (38), ovarian (34, 39, 40), gastric neuroendocrine (41), esophageal (42), and lung (10, 43). Our refinement of the location of two potential tumor suppressor loci now provides an excellent starting point for additional deletion mapping and candidate gene identification.

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