Loss of Heterozygosity Analysis and DNA Copy Number Measurement on 8p in Bladder Cancer Reveals Two Mechanisms of Allelic Loss

Jacqui Adams, Sarah V. Williams, Joanne S. Aveyard, and Margaret A. Knowles

Cancer Research UK Clinical Centre, St. James’ University Hospital, Leeds, United Kingdom

Abstract

Many epithelial tumors show deletion of the short arm of chromosome 8 that is related to aggressive disease or adverse prognosis. In undissected samples of urothelial cell carcinoma of the bladder, at least two regions of loss of heterozygosity (LOH) were identified previously within a small region of 8p11-p12. LOH analysis on a panel of pure tumor DNA samples confirmed this and identified tumors with allelic imbalance, some with clear breakpoints in 8p12. This suggests that these samples contained genetically distinct subclones or that breakpoints in 8p12 may confer a selective advantage without LOH. To assess the mechanism of LOH and to map breakpoints precisely, a panel of bladder cancer cell lines was examined. Microsatellite analysis of 8p markers identified regions of contiguous homozygosity that coincided with regions of LOH in tumors. Fluorescence in situ hybridization analysis was carried out on seven cell lines predicted to have 8p LOH using a chromosome 8 paint, a chromosome 8 centromeric probe, and a series of single-copy genomic probes. This revealed overall underrepresentation of 8p and overrepresentation of 8q. Several breakpoints and one interstitial deletion were identified in 8p12. Two cell lines with extensive interstitial regions of homozygosity showed no reduction in DNA copy number by fluorescence in situ hybridization analysis, indicating that, in addition to large deletions and rearrangements of 8p, small regions of interstitial LOH on 8p12 may be generated by mitotic recombination. This implicates both major DNA double-strand break repair mechanisms in the generation of 8p alterations. (Cancer Res 2005; 65(1): 66-75)

Introduction

Alterations affecting the short arm of chromosome 8 are frequent in human cancers. Most are deletions and these have commonly been identified and mapped by loss of heterozygosity (LOH) analysis, which uses highly polymorphic genetic markers to detect loss of one parental allele in a tumor sample compared with constitutional DNA from the patient. LOH is frequently found in the region of tumor suppressor genes that require biallelic inactivation for effect. This is commonly achieved via LOH and a small mutation in the gene on the retained allele. In bladder cancer, 23% of tumors show 8p LOH, and there is a significant association with muscle invasion (56% of muscle invasive tumors have 8p LOH; ref. 1). 8p LOH is also found in several other tumor types, including prostate and colorectal carcinomas, where it is also associated with a more aggressive clinical phenotype.

The pattern of 8p LOH is complex with at least two regions of deletion (2–6), consistent with the presence of two or more relevant tumor suppressor genes. A distal region of LOH at 8p21-p23 has been identified and mapped in several cancer types, including prostate (7, 8), oral and laryngeal (9, 10), colorectal (11–13), non–small cell lung (13), liver (13), breast (14), and ovarian (15). A second more proximal region on 8p11-p12 shows LOH in many tumor types, including bladder (2), colorectal (16), prostate (3, 4, 6, 17–19), renal (20), breast (21, 22), and oropharyngeal (5). LOH in this region is associated with tumor stage and grade in bladder cancer and nonpapillary renal cell carcinoma (2, 20); with invasive behavior in prostate cancer (3, 18); and with lymphatic, vascular, and perineural microinvasion in colorectal cancer (23).

Two independent regions of interstitial loss of 8p11.2-p12 have been reported in colorectal cancers, suggesting the presence of at least two suppressor genes (16). Homozygous deletions have been found in prostate cancer at D8S87 (8p; ref. 24) and between D8S87 and D8S133 on 8p12-21 (25). Unbalanced chromosome translocations with breakpoints around 8p12, resulting in loss of distal 8p, are reported in breast, colon, and squamous cell lung cancers (26). Breakpoints clustered in a small region around D8S505 in 8p12 have been mapped in three breast cancer cell lines, and in two cases, these were accompanied by additional and complex rearrangements (27).

The 8p12 region is also a site of amplification in breast cancer (28, 29) with a potential target being FGFR1, which maps to 8p12. Coamplification of FGFR1 (8p12) with CCND1 (11q13) has been identified in 5% to 8% of primary breast cancers (28), and in ~3% of primary breast tumors, there is a recurrent rearrangement involving the proximal portions of 8p and 11q resulting in the formation of a hybrid amplified structure composed of 8p and 11q sequences (30).

Functional assays have confirmed the importance of chromosome arm 8p in tumorigenesis. Introduction of a whole human chromosome 8 suppressed invasiveness of a highly metastatic rat prostate cancer (31), with the 8p12-p21 region being necessary for suppression of metastasis (32). 8p12-pter was also found to be critical for suppression of invasiveness in colon carcinoma cells (33). Another study found 8p22-p23 to suppress soft agar clonogenicity in colorectal carcinoma cells (34).

Our previous LOH studies on 193 cases of urothelial cell carcinoma (UCC) of the bladder identified two regions of deletion, a large telomeric region (8p21.1-pter) and a more proximal region at 8p11.2-12 (2). With the recent availability of the correct order for the microsatellite markers used in that study, the more proximal region of deletion is now defined as two separate regions.
Materials and Methods

Tumor Samples. Bladder tissues were obtained by surgical resection from St. James’ University Hospital (Leeds, United Kingdom). All patients gave informed consent. Tissues were snap frozen in liquid nitrogen, embedded in cryoembedding compound (Leica, Milton Keynes, United Kingdom), and stored at −80°C. Fresh frozen blood was used as a source of normal DNA and kept at −20°C until DNA was extracted.

Cell Lines. Fourteen cell lines were used in this study. Thirteen were derived from transitional cell carcinomas: 253J, J82, VM Cub II, J0, N, RT4, UMUC3, T24, HT1197, RT112, SW1710, 5637, and SD. There was one EBV immortalized lymphoblastoid cell line, J82-EBV (kindly provided by Dr. C. O’Toole). All cell lines were grown under standard conditions.

Microdissection of Samples and DNA Extraction. Sections (5 μm) of frozen or paraffin-embedded samples were cut and stained with H&E. For other samples, cells were selectively captured by laser capture microdissection (Arcturus, Mountain View, CA) to minimize normal DNA contamination, and the DNA was extracted using the QIAmp DNA midi kit (Qiagen, Crawley, United Kingdom). DNA from blood was extracted using the Nucleon DNA extraction kit (Nucleon Biosciences, Lanarkshire, United Kingdom), and stored at −20°C until DNA was extracted.

PCR Analysis for Microsatellite Markers on 8p. Genomic DNA was extracted from cell lines using proteinase K digestion and phenol-chloroform extraction. Eighteen chromosome 8–specific microsatellite markers were used. The primer sequences for these markers were obtained from the Genome Database (http://www.gdb.org). For analysis of cell lines, primers were end labeled using T4 polynucleotide kinase and [γ-32P]dATP and PCR reactions were carried out, as described previously (35), using annealing temperatures below the calculated theoretical melting temperature of the primers. The number of alleles seen at each locus was recorded.

For analysis of tumors, the forward primer was fluorescently labeled with either FAM, TET, or HEX dyes. Sequences from tumor DNA and corresponding normal DNA were amplified, the products were denatured and electrophoresed in 4.25% polyacrylamide gels containing 7 mol/L urea, and products were analyzed by Genescan software (Applied Biosystems, Warrington, United Kingdom). Each informative result was repeated at least twice. Allele ratios were visually compared with normal DNA. LOH was scored when the T:N ratio for one allele was ≤0.5.

Fluorescence In Situ Hybridization. Cells were harvested and slides were prepared using standard cytogenetic techniques. Chromosome 8 centromere was detected using a fluorescein-labeled α-satellite probe (Roche, Lewes, United Kingdom) or digoxigenin-labeled D8Z2 (Qbiogene, Illkirch, France). Five cosmide probes mapped to a 7.9-Mb region on 8p12 were selected by screening the LA08NC01 flow sorted chromosome 8 cosmid library (36): 128C10 containing D8S339, 138B12 containing D8S2529, 57F7 containing D8S283, 73E9 containing D8S335, and 129E11 containing D8S135. Two PAC clones from this region, 96P7 containing D8S505 and 124D8 containing D8S87, were provided by Dr. Paul Edwards (University of Cambridge, Cambridge, United Kingdom; ref. 27). BAC clones 155L11, 263C3, 14117, 395H4, 561E1, 529P14, 1612, and 241P12 were obtained from the Sanger Centre and form part of the human genome sequence tiling path (http://www.sanger.ac.uk/HGP/). The relative positions of 8p12 probes and microsatellite loci are shown in Fig. 1. Equivalent distances from 8pter for the other probes are 14117 (26.17-26.34 Mb), 395H4 (25.12-25.31 Mb), 561E1 (24.2-24.4 Mb), 529P14 (21.6-21.8 Mb), 1612 (18.4-18.6 Mb), and 241P12 (9.8-10 Mb). Chromosome 8 paint (Qiogene) and MYC (Qiogene) were used to assess the chromosome 8 and 8q24 content of each cell line.

Probes were labeled with biotin-14-dATP (Life Technologies, Rockville, MD) or digoxigenin-11-dUTP (Roche), denatured, and applied to denatured slides. Commercial probes were hybridized according to the manufacturer’s instructions. Hybridization was allowed to proceed at 37°C for 15 to 40 hours and detected following incubation with FITC-conjugated avidin DCS (Vector Laboratories, Inc., Burlingame, CA) and rhodamine-conjugated anti-digoxigenin (Roche). Slides were mounted in Citifluor (Citifluor Ltd., London, United Kingdom) containing 4’,6-diamidino-2-phenylindole.
Identification of Regions of Interstitial LOH in 8p12 in Bladder Tumor Samples. Fifty-five tumor samples were studied. Ten (18%) showed clear LOH within 8p12 with minimal signal from the lost allele (T:N ratio ≤0.2) as expected for samples of clonal tumors with at least 80% tumor cells. Five tumors showed allelic imbalance within 8p12, which given the purity of samples used implies aneuploidy rather than LOH. Two of these samples showed clear retention of equal copy number of both alleles for at least one marker within the region of allelic imbalance, indicating that a breakpoint was present within the region of interest. Forty-one of the tumors were also analyzed for LOH in 8p21. Three had LOH in 8p21 but not 8p12, and seven had allelic imbalance (T:N ratio 0.2-0.6) in 8p21 but equal representation of both alleles in 8p12, again suggesting breakpoints within 8p. This gives an overall frequency of 23.6% with clear LOH anywhere on 8p and an additional 22% with allelic imbalance. Of the 10 tumors with LOH in 8p12, 6 had loss of all of 8p12 extending distally and 4 had partial loss. These are shown in Fig. 2 together with the two tumors that showed allelic imbalance with breakpoints in 8p12 (tumors 5 and 6). When correct marker order is applied, our previous LOH analysis on undissected samples (2) defined two regions, shown as regions 1 and 2 in Fig. 2. Our current results with pure tumor samples are compatible with this. Only one tumor (tumor 4) had an interstitial region of LOH. This involved a single marker (D8S513) that lies within region 1. Apart from this single case, regions of LOH extended beyond 8p12 toward the centromere or telomere and thus could target distant genes. Nevertheless, these six tumors all showed at least one breakpoint within a relatively small region of 8p. The estimated distance between D8S1791 and D8S283, at the margins of the region containing the breakpoints, is 4.5 Mb based on National Center for Biotechnology Information Build 34 of the human genome.

Identification of Bladder Tumor Cell Lines with Predicted 8p LOH. To determine likely 8p LOH status, we screened 13 UCC cell lines with 18 highly polymorphic microsatellite loci mapping to chromosome 8. For one cell line (J82), a paired immortalized lymphoblastoid cell line was available (J82-EBV) and classic LOH analysis was possible. For the other cell lines, the presence of contiguous regions with single alleles allowed prediction of regions of LOH. Results are shown in Table 1. Lower than expected frequencies of heterozygosity were found at all loci. Two cell lines (T24 and 253J) had one allele at all loci assessed, suggesting loss of an entire parental copy of chromosome 8. Three cell lines (HT1197, JO’N, and HT1376) had only one allele at all 8p loci but two alleles at one of the two 8q loci assessed (D8S84), suggesting loss of one parental copy of 8p with retention of 8q. The remaining cell lines had two alleles at one or more loci on 8p, demonstrating retention of both parental alleles for at least part of 8p. This is illustrated by J82, which had LOH distal to D8S339 when compared with J82-EBV. One allele was seen in ≥11 of the 13 UCC cell lines analyzed at D8S135, D8S283, D8S513, D8S259, D8S339, D8S137, NEFL, and LPL, suggesting these as possible sites of LOH. Differences in observed and expected allele frequencies are shown in Fig. 3.

Six cell lines (SD, RT4, 5637, UMUC3, VMCUB-II, and the lymphoblastoid line J82-EBV), which had two alleles at multiple loci, showed an interstitial region in which several contiguous loci had only one allele. For SD, 5637, and J82-EBV, this included D8S283, D8S513, D8S259, and D8S278 (probability of homozygosity at all four loci 0.00797). VMCUB-II showed one allele at three of these loci, D8S283, D8S513, and D8S259. The cell line RT4 showed a region of homozygosity including D8S282, NEFL, D8S137, D8S339, and D8S278 (probability of homozygosity 0.0001) and UMUC3 showed only one allele at three of these loci (NEFL, D8S137, and D8S339). During our previous LOH analysis of bladder tumors, these markers have all been assessed in the normal DNA of individuals with bladder cancer. In these samples, the expected allele frequencies (Table 1) were observed (data not shown). Based on the allele frequencies, seven bladder cell lines that were predicted to have partial deletion of 8p were selected for FISH analysis. They were RT112, SD, RT4, UMUC3, VMCUB-II, 5637, and J82.

Assessment of Chromosome 8 Content of Bladder Tumor Cell Lines by FISH. The chromosome 8 content of the cell lines was examined using chromosome 8 paint, a single-copy MYC...
probe, and a series of single-copy probes on 8p, which were examined with 8 centromere by two-color FISH (Fig. 4). The cell lines were heterogeneous as shown by varying chromosome 8 copy number. The most common chromosome 8 content for each cell line is illustrated in Fig. 5. The origins of non–chromosome 8 material in markers are known from a multicolor FISH study, which will be described elsewhere (37). For three cell lines, 5637, UMUC3, and RT4, the multicolor FISH study was on clones derived by single cell cloning from the uncloned cell lines used here, and some differences in chromosomes 8 were seen (37).

### Table 1. Allele frequencies on 8p in bladder tumour cell lines.

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NOTE: 1 and 2 represent the number of alleles detected in each cell line (ND, not done).

* The expected heterozygosity values for each marker were obtained from the Genome Database.

† The observed heterozygosity values were calculated as a percentage of tumour cell lines containing two alleles.

§ Imbalanced. Shaded area shows loci with observed heterozygosity at ≤15%. Boxed areas show regions of contiguous interstitial homozygosity.

Figure 3. Frequency of heterozygosity for chromosome 8 microsatellite markers in bladder cancer cell lines. Columns, percentage of the expected frequency of heterozygosity (Genome Database).
J82-EBV was diploid with two apparently normal chromosomes 8. J82 was triploid, with two subclones differing in their chromosome 8 content. Some cells contained one normal chromosome 8, two derivative chromosomes 8, and a derivative chromosome 3, which included part of 8p, whereas the remaining cells had two normal chromosomes 8 and the same two derivative chromosomes 8 but no derivative 3 with 8p (Fig. 4). This resulted in slight gain of 8 from 8pter to 8p12 and loss of two copies from 8p12 to 8p21.3 and one copy from 8p21.3 to 8pter. J82 had breaks on 8p at 8p12 between 128C10 (D8S339) and 263C6 and at 8p21.3 between 561E1 and 343C1. RT112 was diploid with one apparently normal chromosome 8 and two derivative chromosomes 8, resulting in gain of 8q, two copies of 8p11, and loss of 8p from 8p12 to 8pter. RT112 had breaks on 8p at 8p10-11 proximal to 129E11 (D8S135) and at 8p12 between 129E11 (D8S135) and 124d8 (D8S87). SD was triploid with 1 or 2 normal chromosomes 8, 1 or 2 copies of a derivative chromosome 8, a deleted chromosome 8 and a derivative chromosome 20 which included part of 8q. This resulted in gain of all of chromosome 8, except for the small deleted region. VMCUB-II was triplid with three apparently normal chromosomes 8 and a chromosome 8 with a deletion in 8p12 and some cells having four apparently normal chromosomes 8. This resulted in a gain of all of chromosome 8, except for the small deleted region. 5637 was triploid with one apparently normal chromosome 8, two copies of a derivative chromosome 8, and a derivative chromosome 4, which included distal 8p. This resulted in a loss of 8p21.3-22 to 8pter. 5637 had breaks in 8p at 8p21.3-22 between 529P14 and 16I12.

RT4 was tetraploid with three apparently normal chromosomes 8 and one apparently balanced translocation with a breakpoint in 8p22-23.1 between 16I12 and 241P12. There was no loss or gain of chromosome 8 markers in RT4. UMUC3 was hypertriploid with two or three apparently normal chromosomes 8, two derivative chromosomes 8, a deleted chromosome 8, and a derivative 11, which included part of 8q. This resulted in gain of 8q and of distal 8p23. Some of the derivatives were present in only a proportion of cells. UMUC3 had breaks in 8p proximal to 129E11 (D8S135), at 8p22-23.1 between 16I12 and 241P12, and at 8p23 distal to 241P12.

VMCUB-II was triploid with some cells having three apparently normal chromosomes 8 and a chromosome 8 with a deletion in 8p12 and some cells having four apparently normal chromosomes 8. This resulted in a gain of all of chromosome 8, except for the small deleted region. VMCUB-II had breaks in 8p12 either side of the small deletion. 5637 was triploid with one apparently normal chromosome 8, two copies of a derivative chromosome 8, and a derivative chromosome 4, which included distal 8p. This resulted in a loss of 8p21.3-22 to 8pter. 5637 had breaks in 8p at 8p21.3-22 between 529P14 and 16I12.

Thus, five of seven cell lines showed gain of 8q, whereas four showed loss of part of 8p and a further two had part of 8p underrepresented when compared with 8q and 8 centromere. Four cell lines had breakpoints in 8p12 within a region spanning ~9.3 Mb: between 128C10 (D8S339) and 263C6 in J82, between 129E11 (D8S135) and 124d8 (D8S87) in RT112, between 155L11 and 96p7 (D8S505) in SD, and either side of 155L11 in VMCUB-II (Fig. 1). One of the breakpoints in VMCUB-II may be the same as...
Figure 5. Illustration of chromosome 8 content of the cell lines studied by FISH. Pale gray, chromosome 8; dark gray, material from other chromosomes. Probes noted are those closest to the breakpoints but still retained. Asterisk, one or two copies of these chromosomes present in each cell.
that in SD. Three cell lines had breakpoints in 8p10-11, and five cell lines had breakpoints in distal 8p of which three were in the same 9Mb interval between 16I12 and 241P12.

Comparison of Cytogenetic Data with Allele Frequencies. Figure 4 shows the relationship of copy number detected by FISH and regions of predicted LOH in the cell lines. In RT112 and SD, cytogenetically detected losses fitted well with predicted 8p LOH. However, in four of the other five cell lines, there were regions of predicted LOH that were not consistent with cytogenetic observations. In 5637, homozygosity of seven loci from D8S513 to NEFL conferred a high probability of LOH, but this was not accompanied by copy number loss. Five FISH probes within this region showed retention on all three copies of chromosome 8 (Fig. 6). In RT4, five markers with single alleles denoted probable LOH within the region D8S278 to D8S282, but five FISH probes within this region showed retention of all four copies. In VMCUB-II, single alleles were found at D8S259, D8S283, and D8S513, and no loss of two FISH probes containing D8S259 and D8S283, respectively, was detected. The deletion of 155L11 in 80% of cells in VMCUB-II is in a distinct location, separated by the heterozygous D8S535 and D8S505 loci. Similarly, UMUC3 had three contiguous homozygous markers (D8S339, D8S137, and NEFL) but showed no deletion of FISH probes. If, as predicted, the regions of homozygosity in these cell lines represent small interstitial regions of LOH as observed in tumor sample 6, the observed retention of copy number by FISH analysis indicates that LOH was generated via a copy number neutral mechanism such as mitotic recombination (gene conversion).

LOH was found in J82 by comparison with J82-EBV for markers including and telomeric to D8S339. This is compatible with the cytogenetic finding of reduction in copy number of the
FISH probe 263C6. However, the FISH probe containing D8S339 did not show a reduction in copy number, although LOH was recorded for D8S339, possibly indicating that the breakpoint lies within the cosmid probe. Interestingly, J82-EBV was homozygous for four markers (D8S513-D8S278) so that the true extent of LOH could not be determined.

Discussion

This study has revealed considerable complexity in the genomic events that occur on proximal 8p in bladder cancer. We have confirmed by LOH analysis on pure tumor DNA samples that LOH is found in this region in UCC. Although it was not the objective of this study to carry out extensive deletion mapping by LOH analysis, we confirmed the existence of at least two regions of LOH in 8p12-p11 that are compatible with our previous findings (2) and identified one tumor with a very small region of loss. A recent study showed two similar regions of LOH in 8p12 in microdissected breast tumor samples, although the use of different markers precludes direct comparison (38). We found one case with LOH at a single locus only (D8S513). The two critical regions of LOH defined in our previous study (2) lie between D8S335 and D8S278 and between D8S135 and D8S535, and this small region of loss lies within the first of these. The markers that flank this region (D8S283 and D8S535) are only 62.8 kb apart (Ensembl v20.34c.1), and there are no other known polymorphic markers that could be used to confirm that there is true regional LOH. The region contains two Genescan predictions but no known genes. It is possible that a single unidentified gene spans the entire region or that an unrecognized 5′ exon or promoter region of a nearby gene is affected by this event. Further studies of a larger tumor panel will be needed before conclusions can be drawn on the significance of this finding.

An unexpected finding was that, in DNA samples with >80% tumor cell content, some tumors showed allelic imbalance with clear breakpoints within 8p. If these samples contain only one tumor clone, it is difficult to reconcile this finding with the biallelic inactivation of a tumor suppressor gene and equally difficult to explain how such events might confer a selective advantage on the tumor cell. An alternative explanation might be that these samples, albeit morphologically homogeneous, contain more than one evolving subclone. The two tumors with allelic imbalance depicted in Fig. 2 were both grade 3 tumors and might be expected to show a high level of genomic instability. Future studies can address this issue by examining multiple small microdissected samples from such tumors.

A major aim was to define breakpoints in 8p more precisely. Microsatellite analysis of a panel of bladder cancer cell lines indicated frequent reduction to homozygosity in the region of interest. Observed frequencies of heterozygosity for all markers were significantly lower than expected. No cell lines showed heterozygosity for D8S283, which lies within LOH region 1 found in tumors. We predict from allele counts that most cell lines have allelic loss affecting the same region(s) of LOH identified in tumors. Two other regions, one close to D8S339 and the other at D8S135, are also predicted to represent critical regions of allelic loss in these UCC cell lines. Thus, as in tumor samples studied previously (2), there were three distinct regions of predicted allelic loss: one in the region of NEFL, one focused on D8S283, and one more proximal between D8S87 and D8S255. In J82, the only cell line for which paired normal DNA was available, LOH extended from D8S339 to 8pter. Interestingly, the immortalized lymphoblasts from this patient were homozygous at four contiguous markers (D8S513, D8S283, D8S259, and D8S278). Normal tissue from the patient was not available, so it is unclear whether this represents allelic loss that occurred during immortalization of the lymphocytes or the true constitutional genotype of this patient. We have found no evidence for an excess of this haplotype in other patient or normal samples studied.

FISH analysis of seven cell lines with predicted partial LOH of 8p confirmed the presence of frequent underrepresentation of 8p in keeping with several previous cytogenetic reports of monosomy 8, isochromosomes of 8q, and deletions of 8p in bladder cancer, all of which suggest loss of material from 8p and the presence of one or more tumor suppressor genes in the region. The 8p breakpoints identified by FISH are compatible with the regions of LOH found in primary bladder tumors and in other cancers including colorectal cancer and prostate cancer (3, 6, 16). This suggests that the same tumor suppressor gene(s) may be involved in the pathogenesis of these distinct cancers.

Breakpoints in 8p12 were mapped in four cell lines (J82, RT112, SD, and VMCUB-II). This breakpoint region is similar to that identified in three breast cancer cell lines (27), suggesting that this a consistent breakpoint region. The interstitial deletion in VMCUB-II could indicate the location of a gene affected by breaks in this region, although the significance of a loss of one of four copies in 80% of cells is not clear and this is not coincident with the single small interstitial region of LOH in tumor 4. This region also contains two Genescan predictions but no known genes. In J82, RT112, and SD, an affected gene could lie at considerable distance distal to this breakpoint region, as all have a long region of homozygosity extending either to 8pter (J82 and RT112) or to D8S264 (SD).

At least four cell lines, 5637, UMUC3, RT4, and VMCUB-II, had regions of predicted LOH with multiple homozygous microsatellite markers but did not show regional underrepresentation of 8p. This could represent gene conversion, resulting in retention of copy number for a mutated allele, and this might be achieved via mitotic recombination. Homozygosity for seven contiguous markers, which span ~8.8 Mb in 5637, almost certainly indicates LOH and all five FISH markers in the region were retained. We have previously found evidence for this mechanism on 15q, where small, apparently interstitial regions of LOH are found in bladder cancer (39). Analysis of tumor samples must now be carried out to assess the generality of this mechanism of LOH on 8p, but this preliminary information indicates a much greater complexity in the nature of 8p breakage mechanisms than previously expected. Reported studies to date indicate frequent breakage of 8p, commonly associated with translocation and concomitant loss of large regions of 8p. Such observations are compatible with repair of DNA double-strand breaks by the nonhomologous end-joining pathway of repair. However, the LOH detected here in the absence of copy number changes would most probably be achieved via the alternative mechanism of double-strand break repair, homologous recombination.

The region of homozygosity at 8p21 identified in RT4 (including the markers D8S339, D8S137, and NEFL) and UMUC3, which is overlapped by larger regions of homozygosity in other lines, has been identified as a minimal region of LOH in several other cancer types including oral and laryngeal (10), hepatocellular (40), and breast (21). The region containing D8S259, D8S283, and D8S513 has also been implicated in colorectal (16), prostate (3, 6, 24), and breast (37, 41) cancers.
Complex rearrangements and patterns of 8p LOH have been described in other cancer types, but despite great efforts to identify the target gene or genes, to date no good candidates have been identified. Many cancers have LOH involving 8p21-ppter and most genes examined to date have mapped to this part of the chromosome. These include \textit{TNFRSF10B} (DB5), \textit{LZTS1}, \textit{DBC2}, and \textit{DLC1}. Of these, a promising candidate for an 8p21-p22 tumor suppressor gene is \textit{DLC1}, which although not mutated shows significant loss of expression in relevant tumors (42, 43). In 8p12-p11, less attention has focused on possible candidate genes. In bladder cancer, a mutation screen did not identify mutations in \textit{POLB} and \textit{PPPC2B} (44). Mutations of \textit{sFRP1}, an antagonist of Frizzled receptors, which lies centromeric to all probes and markers studied here, have been reported in colorectal cancer (45) but not bladder cancer (46). In breast cancer, deletions, translocations, and complex rearrangements sometimes including amplification proximal to the breakpoints in 8p have been described (27, 47). In a recent study of 43 breast and pancreatic tumor cell lines, in which all but two lines showed chromosome 8 imbalance by CGH and half had 8p loss, seven breakpoints were found within the \textit{NRG1} gene (48). These breakpoints were scattered over a 1.1-Mb region that lies telomeric to the breakpoints described here (Fig. 1). \textit{NRG1} encodes a series of ligands for the \textit{ERBB} family of receptors, including the heregulins and NEU differentiation factors, generated by alternative splicing of 17 exons. In the study of Adelaide et al. (49), mRNA expression of a range of isoforms was examined in cell lines with chromosomal breaks within the gene. In none was expression eliminated, nor was there any consistent pattern of isoform expression in cell lines with breakpoints, so that the relationship of this gene to 8p alterations is not clear. However, the known role for overexpression of receptors for \textit{NRG1} encoded ligands in breast and bladder cancer and the existence of recurrent breakpoints in the gene make this an attractive candidate for future assessment. Indeed, four of the potential interstitial regions of LOH identified here in cell lines include \textit{NRG1}, which lies at the telomeric end of the region examined by FISH.

There is an aphidicolin-induced common fragile site on 8p11-q11 (50, 51), the location of which has not been mapped precisely. If this is on 8p11, it is possible that some of the breaks we have identified have occurred at this site. However, it is clear that many breaks in bladder tumors and cell lines occur in 8p12 or 8p21 where no fragile sites are known. Whatever the mechanism by which these breaks are generated, chromosomal breakage alone is unlikely to account for the finding of 8p loss as a clonal event in specific subgroups of cancers. Selection for loss or breakage in the region clearly indicates that a biological advantage is conferred. It will be important to determine whether fragile sites are involved in these events, as a propensity to breakage at particular sites due to structural features provides no clear information on the likely location of the gene or genes whose loss provides a selective advantage. Indeed, involvement of an unidentified fragile site could lead to inappropriate concentration of efforts on candidate genes in that region.

In conclusion, our study has confirmed that LOH of 8p12-p11 is common in bladder cancer and we have identified several regions involved. The breakpoints identified here in UCC cell lines lie close to breakpoints identified previously in breast cell lines (27), but as they do not target a single gene, it will be important to examine breakpoints in a larger series of cell lines and tumors to determine whether these affect specific genomic motifs. Most importantly, we have shown that two mechanisms may be involved in the generation of genomic alterations on proximal 8p, possibly involving aberrations in two distinct pathways of DNA double-strand repair.

Acknowledgments


Grant support: Cancer Research UK.

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We thank Dr. Paul Edwards for provision of clones for use as FISH probes and for helpful discussions and Carolyn Hurst for help with DNA preparations.

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