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Losses at 3p common deletion sites in subtypes of kidney tumours: histopathological correlations

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Abstract Deletions of the short arm of chromosome 3 (3p) have been recognized as characteristic features of clear cell renal cell carcinomas (clear cell RCC). We analysed 55 clear-cell RCCs and 30 non-clear-cell kidney tumours (10 papillary and 7 chromophobic RCCs, 11 oncocytomas and 2 collecting duct carcinomas) in loss of heterozygosity (LOH) studies using microsatellite markers for previously observed regions of common deletions on 3p in kidney tumours (3p25, 3p21.3, 3p14.2 and 3p12-13). Alterations were found in all 55 cases of clear-cell RCCs at two to four of the 3p regions. Extensive losses were not found in non-clear-cell tumours except for collecting duct carcinomas; 1 of 10 papillary RCCs showed interstitial deletion limited to a single 3p21.3 locus. LOH analyses using microsatellite markers for regions of common deletions at 3p may be of value in differential diagnosis of kidney tumours.

Key words Kidney tumour classification · 3p · Loss of heterozygosity

Introduction

Renal tumours comprise a spectrum of phenotypically and biologically diverse entities, and the distinction between histopathological subtypes such as clear-cell, chromophobic, papillary, sarcomatoid, collecting duct carcinomas and oncocytomas is clinically important. In some cases differential diagnosis can be difficult at the light microscopic level, and it has been suggested that analyses of 3p deletions might be useful in diagnosis. The question of specificity of 3p abnormalities for particular subtypes of renal cell carcinomas (RCC) remains unresolved, although it has been reported that 3p losses are characteristic for clear-cell renal carcinomas [15, 16, 22, 23].

Chromosome 3p loss has been observed in most cases of clear-cell RCC [1, 2, 8, 12, 13, 15, 16, 21–23, 25]. However, in the majority of large series of tumours of this type examined there is always a group of up to 40% of cases in which 3p abnormalities cannot be detected by cytogenetics or loss of heterozygosity (LOH) studies. This may be because not all clear-cell RCCs exhibit 3p losses, because an insufficient density of markers was used to detect small deletions, or because the location of the markers was inappropriate. The probability of detection of losses is highest when markers of common deletion sites are used.

Recently, these regions were delineated in kidney tumours by LOH studies using highly polymorphic microsatellite markers, which improves the possibility of finding 3p losses. Common losses in RCC have been described and delineated at 3p25 (von Hippel-Lindau gene) [5, 7], 3p21 [21, 25], 3p14.2 [region of t(3;8) translocation associated with familial RCC] [5, 12, 21] and 3p12–13 region [12].

In the present study, 55 clear-cell RCCs and 30 non-clear-cell kidney tumours were analysed by LOH studies using microsatellite markers for the aforementioned regions of common deletions on 3p.

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Materials and methods

Samples and DNA extraction

Samples of kidney tumours were obtained from 85 patients immediately after radical nephrectomy (55 clear-cell RCCs, 10 papillary RCCs, 7 chromophobic RCCs, 11 oncocytomas and 2 collecting

Table 1 Clinicopathological findings for kidney tumours

Histological type	Total number	Grade[a]			Stage[b]	
		I	II	III	A	B–D
Clear cell RCC	55	19	17	19	27	28
Papillary cell RCC	10	2	8	–	10	–
Chromophobic RCC	7	3	4	–	6	1
Oncocytoma	11[c]	–	–	–	11	–
Collecting duct carcinoma	2	–	–	2	–	2

[a] According to the method of Thoenes et al. [20]
[b] According to the method of Robson et al. [17]
[c] Not graded

Table 2 Markers used for detection of common deletions at 3p. * Primers labelled with 6-FAM

Marker and its chromosomal position	Primer sequences	PCR conditions
D3S1435 (3p25)	A: 5′ TGGATACATTAGTATACTGAATT 3′ B: 5′*TTAGACGGAAGCAAGGAAGG 3′	94°C, 1 min 58°C, 1 min 72°C, 2 min 25 cycles
D3S1038 (3p25)	A: 5′ TCCAGTAAGAGGCTTCCTAG 3′ B: 5′*AAAGGGGTTCAGGAAACCTG 3′	94°C, 1 min 58°C, 1 min 72°C, 1 min 23 cycles
D3S643 (3p21.3)	A: 5′ GACAGAACTGCCAAACCATCCCAC 3′ B: 5′*TATGTGCTCCAGGCTGGGTAACAG 3′	94°C, 1 min 58°C, 1 min 72°C, 1 min 23 cycles
D3S1295 (3p21.1)	A: 5′ ATTTTATAAGTTTTGATACCCACCC 3′ B: 5′*TGTAGTAATGGTTTCATGGATACAC 3′	94°C, 1 min 58°C, 1 min 72°C, 2 min 24 cycles
D3S1481 (3p14.2)	A: 5′ ATTATACCTCTTTGTAGC 3′ B: 5′*GATGAATATTGTTAGTCC 3′	94°C, 1 min 48°C, 1 min 72°C, 1 min 23 cycles
D3S1284 (3p14.1)	A: 5′ GCCTTGGGGGTAAATACTCT 3′ B: 5′*GGAATTACAGGCCACTGCTC 3′	94°C, 1 min 58°C, 1 min 72°C, 2 min 24 cycles
D3S1274 (3p13)	A: 5′ TTATACATCAGTCTCTGGGAAACAC 3′ B: 5′*TACTGTGCATATAGGTTCCTGTGA 3′	94°C, 1 min 55°C, 1 min 24 cycles

duct carcinomas); histological type, grading and staging for all cases studied are summarized in Table 1. Tumours were carefully dissected on visual inspection to eliminate inclusion of normal tissue before preparation of DNA. Fresh normal kidney tissue from the same patients was also obtained. The tissues were frozen in liquid nitrogen and stored at –80°C. Genomic DNA was extracted according to a standard method involving incubation with proteinase K, phenol/chlorophorm extraction and ethanol precipitation [11].

Primers and polymerase chain reaction

Assessment of allelic deletions by LOH analysis was determined by means of primers that amplify polymorphic microsatellite-containing alleles located within or close to regions of common deletions at 3p [14, 18]. D3S1435 and D3S1038 at 3p25, D3S643 at 3p21.3, D3S1295 and D3S1481 at 3p14.2–21.1, D3S1274 and D3S1284 at 3p13-14.1 were amplified in each normal/tumour DNA pair, and retention or loss of heterozygosity was assessed by comparison of alleles in normal and tumour DNA after polyacrylamide gel separation. Map position and order of the markers were established on the basis of data obtained from the Genome Data Base (Johns Hopkins University) and the Human Cooperative Linkage Center Database and confirmed, where possible, by analysis of a panel of rodent–human hybrids containing portions of 3p [10]. Primer sequences and PCR conditions for individual primer pairs were also obtained from the Genome Data Base. Primers were synthesized on a Model 391 DNA Synthesizer (Applied Biosystems), and one primer in each pair was fluorescently labelled with 6-FAM (Applied Biosystems). The target DNA sequences were amplified by PCR in 12.5 µl final volume containing 1 × Taq buffer (Stratagene); 12.5 pmol of each primer (one fluorescent); 50 µM each of dATP, dCTP, dGTP, and dTTP; 0.5 U Taq polymerase (Stratagene) and 25–30 ng of sample DNA. Reactions were overlaid with mineral oil and the DNA amplified in a thermal cycler (model 480, Perkin Elmer). The cycle number was optimized for each marker to ensure subsaturation PCR conditions (see Table 2).

Polyacrylamide gel electrophoresis

PCR products were analysed on 6% polyacrylamide (Ready Mix DNA/PAGE, Pharmacia) denaturating gels in 1 × TBE buffer in a

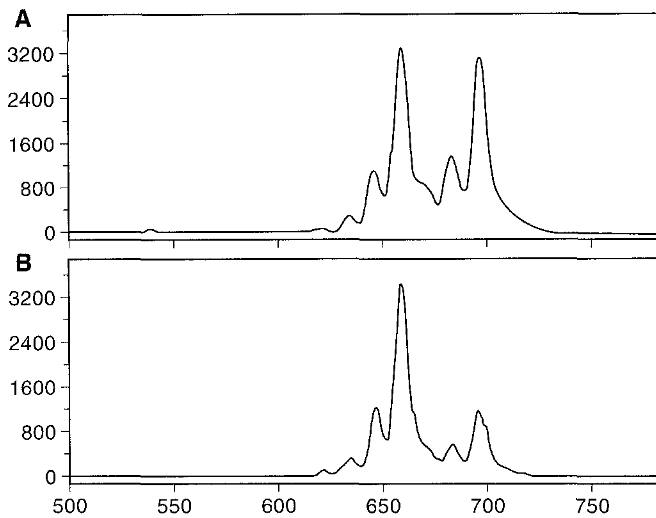

Fig. 1A, B Electrophoretogram of case 101 for marker D3S1038 (3p25). **A** Normal DNA; **B** cancer DNA. An indication of loss of the larger allele in the tumour DNA can be seen from the decrease in height of the peak. Calculation of the allele ratio in this example gives a value of 0.34, which confirms complete loss of heterozygosity (LOH). Scan number in 373A sequencer as a measurement of electrophoretic mobility of PCR products is plotted along the *x*-axis; fluorescent signal (fu) as a measurement of fluorescence intensity of PCR products on the *y*-axis

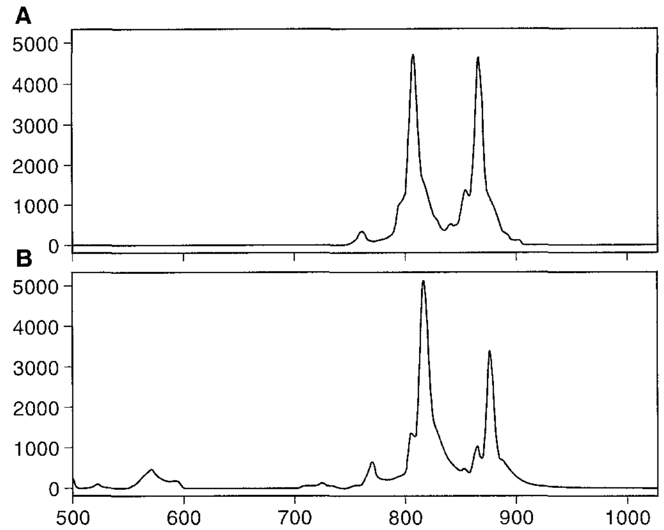

Fig. 3A, B Electrophoretogram of case 101 for marker D3S1295 (3p21.1). **A** Normal DNA; **B** cancer DNA. An indication of loss of the larger allele in the tumour DNA can be seen from the decrease in height of the peak. Calculation of the allele ratio in this example gives a value of 0.7, from which we assumed a partial LOH. *x*-Axis scan number; *y*-Axis fluorescent signal (fu)

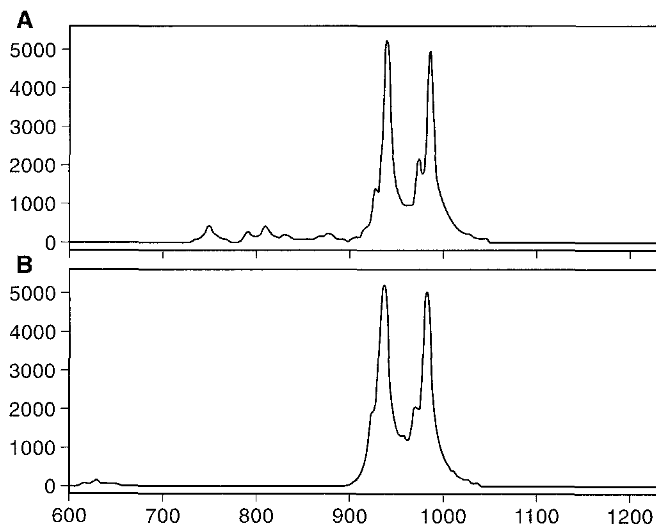

Fig. 2A, B Electrophoretogram of case 101 for marker D3S643 (3p21.3). **A** Normal DNA; **B** cancer DNA. The heights of the peaks referring to both alleles in tumour are similar. Calculation of the allele ratio in this axample gives a value of 0.97, which confirms retention of heterozygosity. *x*-Axis scan number; *y*-axis fluorescent signal (fu)

Model 373A automated DNA sequencer (Applied Biosystems), and 1 μl of each PCR reaction mixture was combined with 3 μl deionized formamide. This mix was denaturated for 5 min at 90°C and, after ice-cooling, 2.5 μl was loaded on the gel. Electrophoresis was for 4 h at 800 V (30 W) and 25°C. During electrophoresis, the fluorescence detected in the laser scanning region was collected and automatically analysed with the GeneScan software (Applied Biosystems).

Calculation of allele ratios

The locus was scored as homozygous when one fluorescent peak was seen in the normal DNA sample. Two peaks were observed when the sample was heterozygous for a marker. The values given for the ratio of peak heights of the two alleles in the paired normal and tumour samples, calculated according to Cawkwell [3], were used to assign a figure for allele loss. In cases showing alterations, PCR analyses were repeated 2 or 3 times. Ratios of 1.00–0.80 for alleles in tumour DNAs were interpreted as retention of heterozygosity, ratios of 0.79–0.51 as partial loss of heterozygosity ("partial deletions") and ratios below 0.51 as complete loss of heterozygosity (see Figs. 1–3 for illustration of fluorescent detection of marker alleles).

Results

In clear-cell RCC, losses within previously identified common regions of deletions at 3p25, 3p21.3, 3p14.2–21.1 and 3p13–14.1 were detected in all 55 cases (for examples of data, see Figs. 1–3, and for summary of data see Fig. 4). Partial deletions were found with markers for 3p25 (case 123); for 3p21.3 (cases 16, 18, 55, 126); and for 3p14.2–21.1 (cases 16, 101, 104, 127). Other loci in these tumours suffered complete loss, as represented by reduction of intensity of one tumour allele by greater than 50%.

Alterations were always observed in at least two of the chromosome regions. In 24 cases we found deletions in all four regions. Losses within 3 and 2 regions (including cases for which one or two of the markers at other loci were non-informative) were detected in 20 and 12 clear-cell tumours, respectively (data not shown).

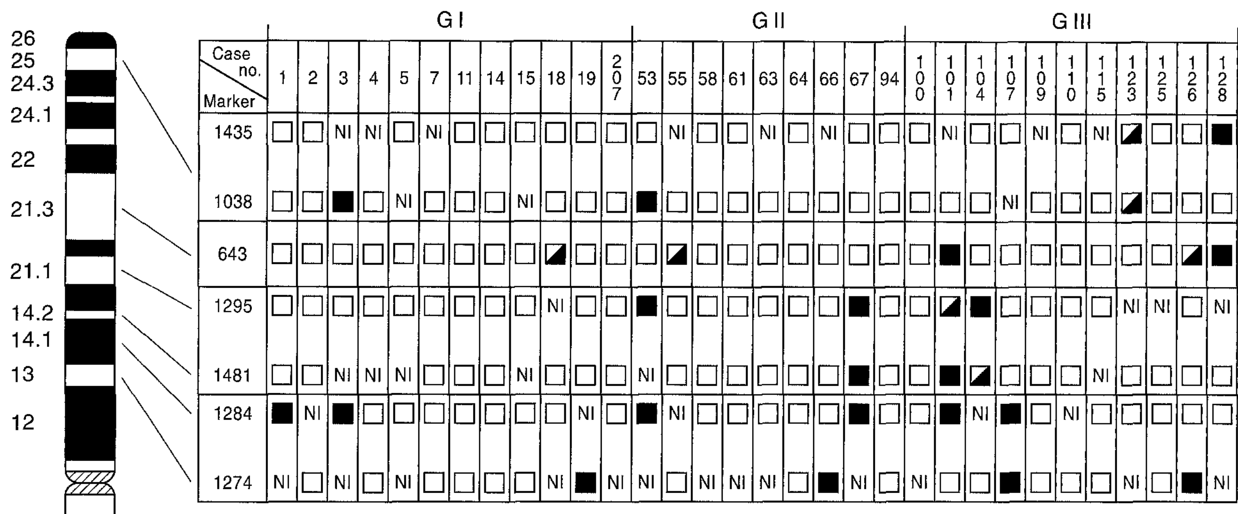

Fig. 4 3p LOH in clear-cell RCCs heterozygous for loci in all four studied regions: ■ Retention of heterozygosity, ◪ partial LOH (signal of one allele reduced in tumor by >20>50%), □ complete LOH (signal of one allele reduced in tumor by >50%), *NI* non-informative

Table 3 Number of clear-cell RCCs with two, three or four deleted regions in relation to clinicopathological findings

Classification	LOSS[a]		
	2 regions	3 regions	4 regions
Grade			
I	1	2	9
II	2	–	7
III	–	3	8
Stage			
A	2	3	11
B–D	1	2	13

[a] Tabulation includes only cases of clear-cell RCC that exhibited heterozygosity at loci within all four regions

Heterozygosity at all of the four regions was observed in 32, cases as illustrated in Fig. 4. In this group, deletions occurred at two regions in 3 tumours (cases 3, 53, 67) and at three regions in 5 (cases 1, 19, 101, 107, 128). Losses limited to 3p21.3 and 3p25 were detected in cases 53 and 67. In case 3, deletions were found only with markers for 3p21.3 and 3p14.2–21.1. Among cases showing losses at three regions while retaining the fourth, alterations occurred at 3p25, 3p21.3 and 3p14.2–21.1 in 3 cases (1, 104, 107) or at 3p25, 3p14.2–21.1 and 3p13–14.1 in 2 cases (101, 128). Frequencies of clear RCCs with two, three and four deleted regions relative to the number of cases and clinicopathological findings are displayed in Table 3. From this summary, it appears that the number of deleted regions in particular cases is not correlated with tumour grade or stage.

None of the 7 chromophobic carcinomas and 11 oncocytomas studied showed 3p deletions. Similar results were obtained in 10 papillary tumours with 1 exception, a case with an apparent interstitial deletion limited to the 3p21.3 region (data not shown).

In both collecting duct carcinomas, losses occurred at 3p25 and 3p21.3; in one of them an additional deletion was detected with marker 1481 at 3p14.2, as summarized in Fig. 5.

Discussion

It has been recognized that accumulation of genomic abnormalities, including losses of tumour suppressor genes, is a crucial event in carcinogenesis [24]. Deletions of 3p have been found to be a characteristic feature of clear-cell RCC [1, 2, 8, 12, 13, 15, 16, 21–23, 25], but we suggest that even more characteristic of this type of tumour is the accumulation of 3p losses. Using highly polymorphic microsatellites, we found alterations in 100% of clear-cell RCCs in at least two of the four regions of 3p for which common allele losses have previously been described for kidney and other tumour types. Accumulations of losses were not found in non-clear-cell tumours except for collecting duct carcinomas.

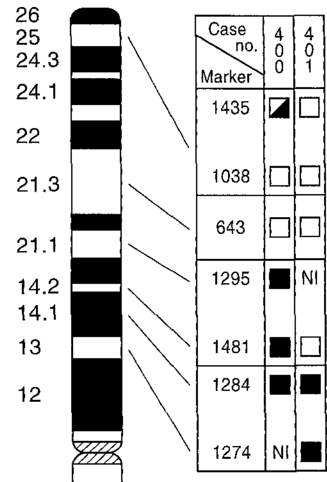

Fig. 5 3p LOH in cases of collecting duct carcinoma. Symbols as for Fig. 4

Our results indicate that clear-cell kidney tumours require accumulation of alterations in two or more of any of the target genes within the regions of common deletions at 3p25, 3p21.3, 3p14.1–21.1 or 3p12-13. No doubt the *VHL* gene is the target of 3p25 loss [5, 7], but the other target genes have not yet been identified. The probability of involvement of particular loci does not seem to be significantly different. The order in which the specific loci are lost or altered is variable, as suggested by analyses of cases in which some regions are not deleted or show only partial LOH.

Those tumours in which complete LOH was observed are likely to be clonal. Partial LOH was found with some markers in a few tumours, and it seems that this finding is not the result of admixture of normal cells with the tumour cells, because we found complete LOH with some markers in the same DNA samples and only partial LOH using other probes. Working under PCR subsaturation conditions and using the GeneScan system, which can detect quantitative changes in signal intensity objectively, we found that results of LOH analyses were highly reproducible. Additionally, on histological slides made from the same parts of the tumour as DNA had been isolated from we found no more than 5–10% of normal cells. Partial LOH can thus be interpreted as the occurrence of 3p abnormalities in subclones of malignant cells.

The observed losses may result from interstitial deletions or allelic losses of large portions of chromosomal arms. The pathogenesis of kidney tumours at the molecular level certainly merits further investigation, but whatever the mechanism of observed alterations the most interesting point from the diagnostic point of view is that the range of 3p abnormalities detected by LOH is very characteristic for clear-cell RCC.

Collecting duct carcinomas also showed extensive 3p losses in both cases studied. However, our results are not in agreement with others reported. Deletions of 3p were not detected by LOH analysis in two cases studied by El-Naggar [4] and 3p alterations were not observed cytogenetically in three cases described by Füzesi [6].

The published data on chromophobic carcinoma are not consistent. Van den Berg et al. studied two such tumours, and no 3p deletions were found [21, 23], which is in agreement with our results. Kovacs et al., however, reported loss of constitutional heterozygosity at 3p in five of nine chromophobic tumours [9].

In 1 of 10 papillary carcinomas in our series we found an isolated loss at a single locus. This is in agreement with data in the literature; interstitial deletions at single loci, rather than more extensive 3p alterations, were previously reported not only on clear-cell carcinomas but also in exceptional cases of papillary tumours [16, 21] and oncocytomas [16, 19, 21].

In summary, extensive allelic losses in 3p25, 3p21.3, 3p14.2 and/or 3p13-14.1 regions seem to be characteristic for clear-cell carcinomas. LOH analyses using microsatellite markers for regions of common deletions at 3p might be of value in the differential diagnosis of kidney tumours. The practical diagnostic utility of our findings should be confirmed by studies of larger series of clear- and non-clear-cell tumours, including cases that are difficult to diagnose at the light microscopic level.

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