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Original Paper

Genomic Aberrations in Renal Cell Carcinomas Detected by Restriction Landmark Genomic Scanning

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In order to reveal and characterise genetic events occurring in renal tumorigenesis, samples of sporadic renal cell carcinomas (RCCs) were examined using restriction landmark genomic scanning (RLGS), an electrophoretic separation technique which detects gene amplification and deletion. We were able to find two fragments frequently amplified and 10 others commonly showing reduced signal intensity within the 16 tumour samples analysed. These altered spots were located on chromosomes 2, 3, 9–12, 16, 17 and 18 according to chromosomal assigned RLGS. A subset of reduced fragments appeared to be correlated to tumour type and were located within a new chromosomal region, suggesting genetic specificity within the process of renal carcinogenesis. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

RENAL CELL carcinoma (RCC) is the most common tumour of the mature kidney, causing more than 1500 patient deaths per year in Japan. In spite of numerous investigations, the aetiology of the disease is still poorly understood. Previous conventional cytogenetic studies have focused on chromosome 3p in RCC development [1–7], in that loss of heterozygosity (LOH) commonly occurs in three separate regions of chromosome 3p. One of these regions, 3p25-26, has been recently identified as the *VHL* gene; it has shown frequent deletions and/or other mutations in sporadic RCC [8, 9]. The region 3p12-14 contains a breakpoint often found in familial RCC, and still another location, 3p21, appears to be deleted in some sporadic cases. At present, the genetic aberration of the *VHL* gene is believed to be specific to non-papillary clear cell tumours and it is, thus, postulated that other histological types of RCC may require alterations at different sites and different genes.

Part of the difficulty in determining the aetiology of the various types of RCC may be due to the restrictions of earlier analytical methods, which could examine only small segments of genes at a time by means of specific primers and probes. Restriction landmark genomic scanning (RLGS) is a recently developed method of surveying essentially the entire genome via the judicious use of specific restriction enzymes and two-dimensional electrophoresis of the resulting fragments. The result is an autoradiographic profile of approximately 2000 spots corresponding to characteristic fragments of DNA cut at landmark sites, the intensities of which reflect copy number [10]. It is a highly reproducible analysis conducive to comparison between tumour and normal tissue DNA for the identification of gene amplification and deletion [11, 12]. In addition, when a methyl-sensitive restriction enzyme, such as *Not*I, is used, RLGS allows for the determination of the methylation status of critical sites [13]. The chromosomal origin of the majority of *Not*I fragments in RLGS profiles has been previously determined by analysis of DNA from flow-sorted human chromosomes [14].

In previous studies on human gliomas and prostate cancers using RLGS, we were able to detect common genetic alterations in malignant tumours, but not in benign lesions [15–17]. Since it is generally accepted that tumorigenesis is a progressive process likely to involve multiple genetic/molecular insults [2, 6], we undertook to examine samples of RCC, not only for the presence or absence of common

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genetic alterations, but also for events related to progression and tumour type. Therefore, we further analysed LOH within the *VHL* gene in the same RCC samples using Southern blot hybridisation and compared the results with those produced by RLGS. Our data indicate some potentially characteristic genetic anomalies in RCC, which may also be correlated to histopathological features.

MATERIALS AND METHODS

Tissue samples

Primary tumours and corresponding normal kidney tissues were obtained from 16 patients with RCC undergoing radical nephrectomy at Nara Medical University Hospital or affiliated hospitals. We received informed consent from each patient for the analysis of DNA in tissue samples.

The tissue samples were snap-frozen in liquid nitrogen and frozen sections from the samples were fixed in formalin and paraffin-embedded for histopathological diagnosis. The adjacent frozen slices to the haematoxylin and eosin sections were used for DNA extraction. Tumours were characterised according to the modified WHO classification [18]; being classified as solid, alveolar, tubular, cystic or papillary by histological pattern, and as clear, granular, mixed, pleomorphic or spindle by cell type. Staging and grading were carried out according to the tumour-node-metastasis (TNM) classification system [19] and the WHO grading system [20], respectively.

DNA extraction

Frozen tissue samples, weighing approximately 0.2–0.3 g each, were ground, suspended in 2 ml proteinase K buffer (500 mM/l ethylene diamine tetraacetic acid (EDTA), 10 mM/l Tris–HCl and 0.5% Sarcosyl) containing 1 mg/ml RNase (Sigma type II, Sigma Chemical Co., St. Louis, Missouri, U.S.A.) and incubated at 37°C for 20 min. Proteinase K was added to give a final concentration of 1 mg/ml and the mixture incubated at 65°C for 1 h. High molecular weight genomic DNA was isolated from each sample after phenol/chloroform extraction and ethanol precipitation.

Southern blot hybridisation

An aliquot of each tumour and control DNA sample was cleaved with the restriction enzyme, *Pst*I (Takara Shuzo Co., Shiga, Japan), electrophoresed in 0.8% agarose gels and transferred to nitrocellulose membranes. The membranes were then hybridised with 1.65 kb of cloned g7-11 cDNA from the *VHL* gene (kindly provided by Dr B. Zbar, National Cancer Institute, NIH, Bethesda, Maryland, U.S.A.) labelled via a random priming procedure [21]. Washing and autoradiography were performed as previously described [22].

RLGS

Sample analysis by RLGS followed the standard protocol detailed previously [10, 11]. In brief, 10 μg of extracted genomic DNA from each tissue sample was treated with 10 U DNA polymerase I (Takara Shuzo) in the presence of 0.33 μM/l dGTPαS, 0.33 μM/l dCTPαS, 33 μM/l ddATP and 33 μM/l ddTTP. Sample DNA was then digested with 100 U of *Not*I (New England Biolabs, Beverly, Massachusetts, U.S.A.). The cleaved ends thus generated were filled in with 20 U Sequenase, Version 2.0 (U.S. Biochemical, Cleveland, Ohio, U.S.A.) in the presence of 0.33 μM/l [α-^{32}P] dGTP (3000 Ci/mM) and 0.33 μM/l [α-^{32}P] dCTP (6000 Ci/mM). A second digestion was then performed using *Eco*RV (Takara Shuzo); the resulting fragmented DNA was loaded on to a 0.8% Seakem GTG agarose gel (FMC Biological, Rockland, Maine, U.S.A.) and electrophoresed at 8 V/cm for 12 h. A third digestion was carried out on the DNA *in situ* with 1500 U *Hinf*I (New England Biolabs) and that part of the Seakem gel containing the DNA digest was excised and fused to a second polyacrylamide gel. A second electrophoresis was performed on the fused gels by applying 8 V/cm for 7 h in a direction perpendicular to first agarose separation. The gels were dried and exposed to X-ray film (Kodak X-Omat, Eastman Kodak, Rochester, New York, U.S.A.) for 3–14 days with intensifying screens.

Analysis of RLGS profiles and statistical tests

Profile analyses were performed using the PDQUEST software program (PDI Inc., Huntington Station, New York, U.S.A.), which matches selected fragments or spots in paired RLGS autoradiographs and assigns a relative score based on internal normalisation to the control. Normal renal tissue from the same patient was used as the control for every profile analysis. The intensities of spots selected for comparison in tumour profiles can thus be expressed as *x*-fold enhancement or reduction of the value of corresponding control intensity, which was denoted as 1.0. Values of 2.0, therefore, indicate 2-fold amplification, while LOH or complete allelic deletion is implied by values of 0.5 or 0, respectively.

To determine the chromosomal location of selected altered spots, we compared our results with those obtained in correlated chromosomal assigned RLGS [14].

Fisher's exact probability test was used to determine statistical significance. Probability values < 0.05 were considered significant.

RESULTS

A compilation of patient sex and age and the histopathological characteristics of their tumours is presented in Table 1. The average age of patients was 60.9 years with a range of 45–79 years.

Every tumour had more than 100 altered intensity spots and most of them were sporadic or infrequent (data not shown). With the exception of the single spindle cell lesion (case 16), one or other of two distinct spots, designated A1 and A2, were found to be amplified in all samples (Table 2; Figures 1 and 2); two tumours, in fact, showed amplification of both fragments (cases 1 and 3). A1 was amplified in seven (44%) of the RCCs with more than a 4-fold mean increase in intensity (data not shown). In conjunction with chromosomal assigned RLGS studies, spot A1 appears to be located on chromosome 16. However, the location of A2, which was amplified in 11 (69%) of the tumours, could not be determined by chromosomal analysis.

Ten spots were frequently reduced in intensity throughout the samples. Designated as R1–R10, at least one of the 10 showed reduced signal intensity in all tumours at incidences ranging from 25% for spot R3 to 81% for R10 (Table 2); spots R1 and R10 may prove to be characteristic of RCC, observed in 69% and 81% of the samples analysed. In most instances, the signal was diminished by approximately half that of the comparable control, which may be indicative of LOH. However, signal strengths of five of these spots were decreased to undetectable ranges in some samples: spot R1 could not be detected in tumours 14, 15 and 16; spot R10

Table 1. *Patient tumour characteristics*

Case	Age (years)	Sex	Stage*	Grade†	Histological pattern‡	Cell type‡
1	64	M	pT2N0M0	1	Alveolar	Clear
2	70	M	pT2N0M0	1	Alveolar	Clear
3	54	M	pT2N0M0	2	Papillary	Granular
4	63	M	pT2N0M0	1	Alveolar	Clear
5	45	M	pT2N0M0	2	Alveolar	Clear
6	79	F	pT3bN0M0	2	Tubular	Clear/granular
7	63	F	pT3aN0M0	2	Alveolar	Clear
8	62	M	pT2N0M0	3	Tubular	Clear/granular
9	51	M	pT1N0M0	2	Tubular	Granular
10	63	M	pT2N0M0	2	Alveolar	Clear
11	50	M	pT2N0M0	2	Alveolar	Clear/granular
12	68	M	pT3aN0M0	2	Tubular	Clear/granular
13	66	M	pT3bN0M1	2	Tubular	Granular
14	56	M	pT3aN0M0	2	Papillary	Granular
15	62	M	pT3bN0M0	3	Solid	Clear/granular
16	59	M	pT4N2M0	3	Solid	Spindle

*According to the tumour-node-metastasis (TNM) classification system [19]. †According to the WHO grading system [20]. ‡According to the modified WHO classification system [18].

was not seen in lesions 6, 8 or 11; spot R4 was lost in the profile of tumours 13 and 16. Two other RCCs showed undetectable R5 and R6 (cases 12 and 13, respectively). No detectable signal is interpreted as complete alleleic loss.

The chromosome number determined by adjunctive chromosomal assigned RLGS studies for each of these reduced fragments is additionally listed in Table 2. As can be seen, the chromosome location for spots R1 and R10 could not be assigned. This may be due to the fact that an immortalised human lymphoblast cell line, GB150, was used in the chromosomal analysis and R1 and R10 may be specific to kidney tissue. The chromosome number for R3 was also not specified, although it is believed to lie on 9, 10, 11 or 12; chromosomal assigned RLGS was not able to differentiate between these chromosomes.

When comparing the profiles against sample histopathology, we found that fragment R8 showed reduced intensity only in clear cell or mixed clear–granular cell tumours at frequencies of 83% and 40%, respectively. Decreases in spots R3 and R7 appeared to correlate with tumour grade and stage. R3 was reduced in 100% of grade 3 lesions (3/3) and in a single grade 2 tumour (1/10), while a reduction in R7 intensity appeared to be a feature of pT3 and 4 stage carcinomas exclusively (7/7, 100%). These results are summarised in Table 3.

We also probed for LOH in the g7-11 region of the *VHL* gene in all RCCs using Southern blot hybridisation. Most of the tumour samples proved to be uninformative. However, of the three cases giving measurable results, LOH was found in only one tumour (case 6) (Table 2). All three of these

Table 2. *Alteration of spot intensities in renal cell carcinoma (RCC) by restriction landmark genomic scanning (RLGS)*

	Spots												Loss of heterozygosity of the *VHL* gene
Case	A1	A2	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	
1	●	●	○	○	–	–	–	–	–	–	○	–	N
2	–	●	○	○	–	○	–	○	–	○	–	○	N
3	●	●	–	–	–	–	–	–	–	–	–	○	N
4	–	●	–	–	–	○	○	–	–	○	–	○	R
5	–	●	–	–	–	○	–	–	–	○	○	○	N
6	–	●	○	–	–	○	○	○	○	○	○	◎	L
7	●	–	–	–	–	–	–	–	○	○	–	○	N
8	–	●	○	○	○	○	○	○	–	–	○	◎	N
9	–	●	–	–	–	–	–	–	–	–	○	–	N
10	●	–	○	○	–	–	–	–	–	○	○	○	N
11	●	–	○	–	–	○	–	–	–	○	○	◎	N
12	–	●	○	○	–	○	◎	–	○	–	–	–	N
13	–	●	○	○	○	◎	○	◎	○	–	○	○	N
14	●	●	◎	–	–	–	–	○	○	–	–	○	N
15	●	–	◎	○	○	–	–	○	○	-	○	○	R
16	–	–	◎	○	○	◎	–	–	○	–	○	○	N
Chromosome no.	16	ND	ND	2	9–12	18	2	17	18	3	3	ND	
Incidence of alteration (%)	44	69	69	50	25	56	31	38	44	44	63	81	

Each spot intensity in RCCs is expressed as fold enhancement against that in the corresponding normal kidney tissues. Each spot has been assigned to the chromosome by a chromosomal assigned RLGS study. ●, increase (intensity > 2.0); ○, decrease (intensity ≒ 0.5); ◎, decrease (intensity ≒ 0); –, no alteration; N, not informative; R, retention of heterozygosity; L, loss of heterozygosity; ND, not determined.

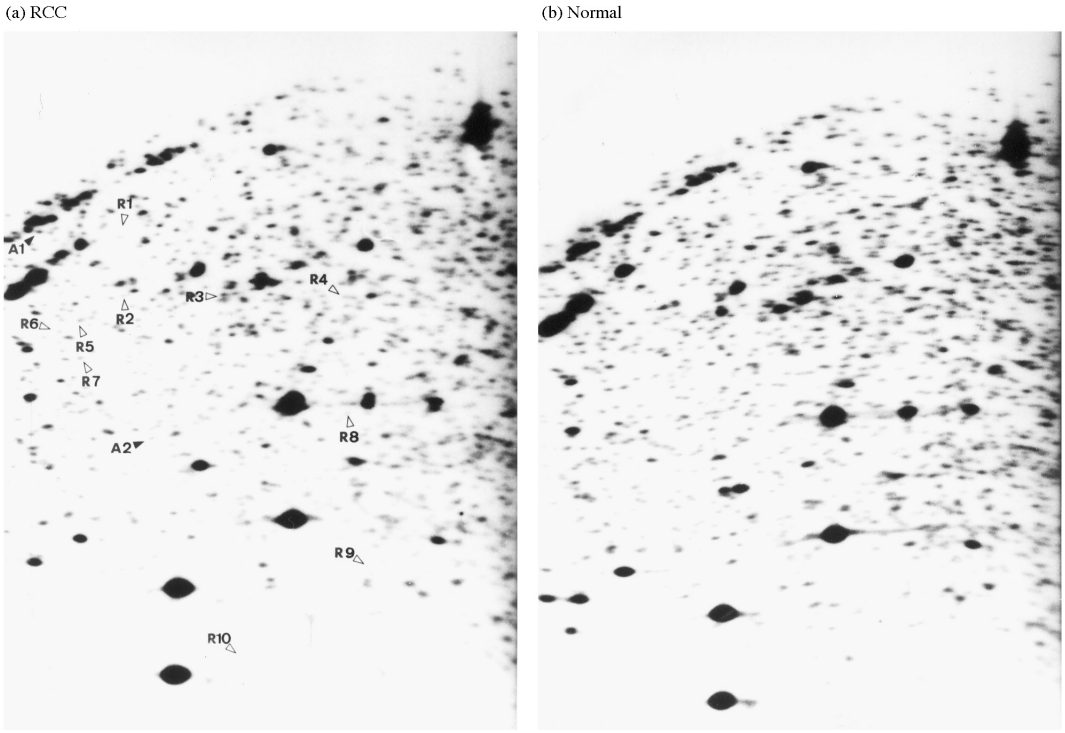

Figure 1. Representative restriction landmark genomic scanning (RLGS) profiles of (a) renal cell carcinoma (RCC); and (b) the corresponding normal kidney tissue from case 14. Altered spots were detected by comparison with normal tissue profiles. Twelve altered spots consisting of two increased spots (black arrowhead) and 10 decreased spots (white arrowhead) were frequently observed in RCCs.

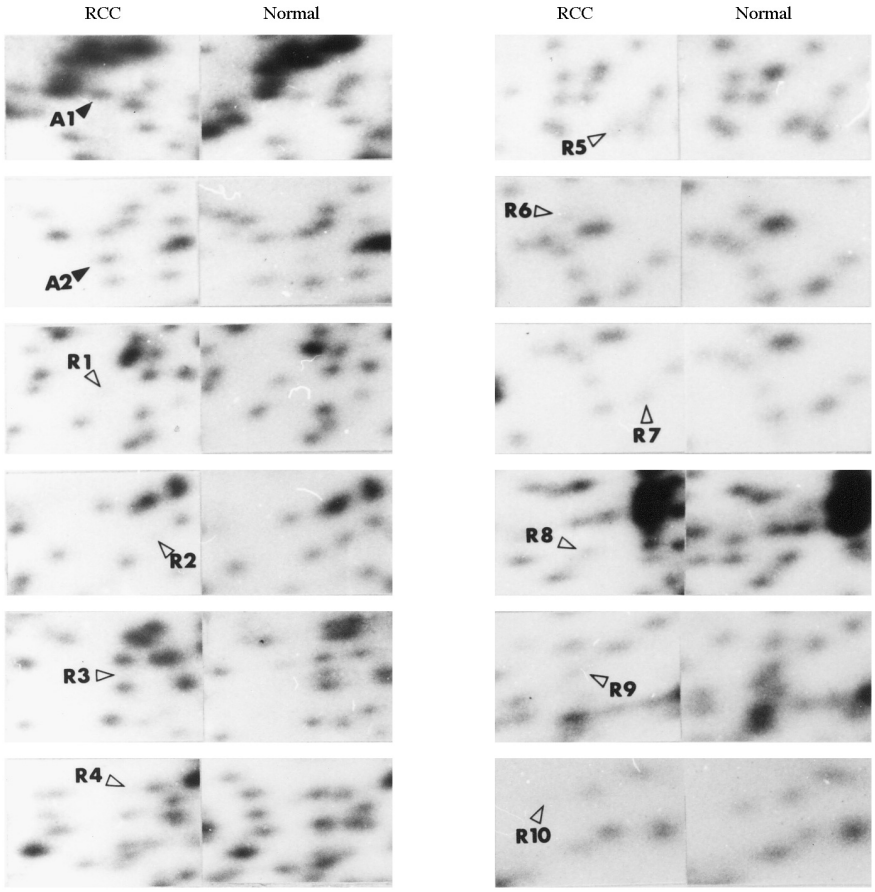

Figure 2. Magnified view of the 12 altered spots. Spots A1 and A2 showed increased intensities, while spots R1–R10 showed decreased intensities.

Table 3. Correlation between spot alteration and histopathological features

Spot (chromosome no.)	Incidence				Statistics
	Clear	Granular	Clear/granular	Spindle	
R8 (3)	5/6 (83%)	0/4 (0%)	2/5 (40%)	0/1 (0%)	$P = 0.015$*
	pT1	pT2	pT3	pT4	
R7 (18)	0/1 (0%)	0/8 (0%)	6/6 (100%)	1/1 (100%)	$P = 0.001$†
	G1	G2	G3		
R3 (9–12)	0/3 (0%)	1/10 (10%)	3/3 (100%)		$P = 0.007$‡

Fisher's exact probability test was used. *Clear cell subtype versus non-clear cell subtype. †pT1,2 versus pT3,4. ‡G1,2 versus G3.

tumours—cases 4, 6 and 15—showed reduced signal of spot R8 which was determined to reside on chromosome 3, and, in cases 6 and 15, R8 intensity correlated with *VHL* gene heterozygosity. In case 4, it did not (Figure 3; Table 2). We therefore attempted to confirm the association between spot R8 and the *VHL* gene by examining whether or not the VHL probe hybridised directly with R8 DNA extracted from the RLGS gels. Hybridisation was not observed (data not shown).

DISCUSSION

RLGS is a methodology developed to examine simultaneously many loci within the genome using particular radiolabelled restriction enzymes rather than specific tailored probes. For example, there are approximately 4000 *Not*I restriction sites within the vertebrate genome; when *Not*I is used, approximately 1000, or 25%, of those sites in genomic DNA become landmarks for screening in a single gel [12]. Further DNA cutting by other selected enzymes produces more landmarks for evaluation. It has been shown to be an extremely sensitive assay, detecting as little as a 2-fold amplification [11]. RLGS additionally provides the ability to detect LOH and homozygous deletions via the differentiation of DNA ploidy at particular loci, and can be used to investigate the methylation status at relevant genetic locations. Again using digestion with *Not*I as an example, demethylation at a *Not*I site produces a novel signal in an RLGS profile, since the enzyme is methyl-sensitive and will not normally cleave a methylated site [12]. As long as the same set of restriction enzymes are used, RLGS profiles are highly reproducible and comparable among individuals of the same species [12], and the technique, therefore, is an accurate and useful tool for investigating common genetic or epigenetic aberrations in cancer.

In the current investigation, the signal intensities of two fragments/spots were amplified in 15 of the 16 samples (94%)

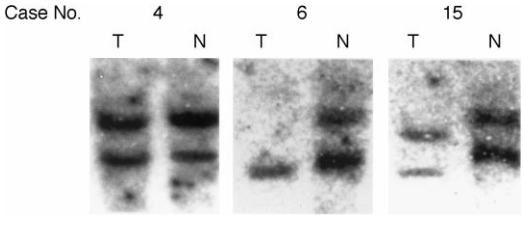

Figure 3. Southern blot hybridisation with the VHL probe (g7-11). Of 16 renal cell carcinomas (RCCs), only three tumours (cases 4, 6 and 15) were informative. Case 4 and case 15 showed retention of heterozygosity, while case 6 showed loss of heterozygosity. T, tumour; N, normal tissue.

surveyed. The enhanced intensity of spot A1 is likely to reflect gene amplification, while the increase in spot A2 signal is proposed to be the result of a *Not*I site demethylation, since this spot was not apparent in the RLGS profiles of correlated normal tissues. A1 was assigned to chromosome 16 by chromosomal assigned RLGS. A conventional cytogenetic study has demonstrated trisomy of chromosome 16 occurring in 48% (36/75) of RCC examined [1], but gene amplifications at this location have not previously been reported in renal carcinoma. Comparative genomic hybridisation has recently revealed gains of chromosome 16 in 27% (4/15) of renal cancers analysed by this technique [23]. The higher incidence of chromosome 16 anomalies found in the present study is probably due to the differing sensitivities of RLGS and comparative genomic hybridisation; in order to detect chromosome gain, comparative genomic hybridisation requires alteration of more than 10 Mb-p [24], but RLGS can theoretically detect amplicons of less than 100 kb-p [11].

We also discovered 10 spots reproducibly decreased in intensity in RCC. Except for those on chromosome 3, the incidences of signal decrease were higher overall than in previous LOH or comparative genomic hybridisation analyses [3–6, 23–26]: Spots R2 and R5 on chromosome 2 were reduced in 50% and 31% of tumours, respectively; R8 and R9 on chromosome 3, 44% and 63%, respectively; R6 on chromosome 17 was reduced in 38% of tumours; R4 and R7 on chromosome 18, 56% and 44%, respectively. The greater numbers of decreased spots detected indicate new loci affected in renal tumorigenesis.

The possibility, however, that these reduced spots reflect methylations at *Not*I sites, rather than loss of alleles, cannot be ruled out. Approximately 89% of all *Not*I sites in the human genome exist within CpG islands lying in the 5′ region of genes and, except for genes concerned with imprinting or those on the inactive X chromosome, they are usually hypo- or completely unmethylated [27–29]. Methylation of CpG islands is thought to play an important role in the regulation of transcription. 5′ CpG methylations of the tumour suppressor genes *Rb*, *p16/CDKN2* and *VHL*, as well as the cadherin genes, have recently been reported to occur in various human malignancies [30–33] and are thought to be associated with the inactivation of such genes. Thus, the decrease in signal intensities of the 10 spots in this study may be indicative of gene inactivation as well as gene deletion.

A comparison of spot alterations on RLGS films with the histopathological features of each tumour identified some aberrations that appeared to be specific to cell type, grade and stage. Spot R8, located on chromosome 3, showed reduced intensity in clear and mixed clear–granular cell neoplasms, but no characteristic alteration was evident for purely

granular cell lesions. LOH of the *VHL* gene, also located on chromosome 3, was demonstrated in one mixed carcinoma showing decreased R8 signal; in another tumour, however, reduced R8 did not correlate with LOH, and further probing of R8 DNA isolated from RLGS gels with the *VHL* gene gave no positive hybridisation results. This implies R8 is in a region different from that of *VHL*. Spot R3 showed reduced signal intensity in all three grade 3 tumours and in a single grade 2 lesion. Stage pT3 and 4 carcinomas showed a specific decrease in fragment R7 on chromosome 18. Tumour progression in other types of cancers has been linked to LOH on 18q [34, 35] and the *DCC* gene, which is implicated to be a tumour suppressor gene, has been mapped to 18q21 [36]. However, LOH of the *DCC* gene is infrequently reported in renal cancer [26, 37], suggesting that R7 may be a separate novel suppressor gene.

RLGS profiles from previous studies on other cancers show that two reduced spots found in RCCs, R1 and R5, are also apparent in hepatocellular carcinoma (HCC); decreased R1 was observed in 69% (11/16) of HCCs [12], while R5 was evident in 75% (12/16) of HCCs [12] and in three of three hepatoblastomas [38]. These genomic events are thus likely to be features of tumorigenesis in general, but it is interesting to note that neither of the two fragments commonly amplified in RCCs, A1 and A2, were evident in the RLGS profiles of prostatic cancers, gliomas, HCCs or hepatoblastomas [12, 15, 16, 38].

The data generated by this investigation indicate the presence of genetic/epigenetic aberrations specific to RCCs, particularly those expressed as amplification of spot A1 and A2 and reduction of R1 and R10. These four fragments are proposed to play roles in neoplastic development, while alterations in spots R3 and R7, although not appearing as frequently, appear to be associated with RCC progression. The cloning of these individual fragments is now in progress.

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