Role of chromosome 3p12–p21 tumour suppressor genes in clear cell renal cell carcinoma: analysis of VHL dependent and VHL independent pathways of tumorigenesis

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Abstract

Aims—Chromosome 3p deletions and loss of heterozygosity (LOH) for 3p markers are features of clear cell renal cell carcinoma but are rare in non-clear cell renal cell carcinoma. The VHL tumour suppressor gene, which maps to 3p25, is a major gatekeeper gene for clear cell renal cell carcinoma and is inactivated in most sporadic cases of this disease. However, it has been suggested that inactivation of other 3p tumour suppressor genes might be crucial for clear cell renal cell carcinoma tumorigenesis, with inactivation (VHL negative) and without inactivation (VHL positive) of the VHL tumour suppressor gene. This study set out to investigate the role of non-VHL tumour suppressor genes in VHL negative and VHL positive clear cell renal cell carcinoma.

Methods—Eighty two clear cell renal cell carcinomas of known VHL inactivation status were analysed for LOH at polymorphic loci within the candidate crucial regions for chromosome 3p tumour suppressor genes (3p25, LCTSGR1 at 3p21.3, LCTSGR2 at 3p12 and at 3p14.2).

Results—Chromosome 3p12–p21 LOH was frequent both in VHL negative and VHL positive clear cell renal cell carcinoma. However, although the frequency of 3p25 LOH in VHL negative clear cell renal cell carcinoma was similar to that at 3p12–p21, VHL positive tumours demonstrated significantly less LOH at 3p25 than at 3p12–p21. Although there was evidence of LOH for clear cell renal cell carcinoma tumour suppressor genes at 3p21, 3p14.2, and 3p12, both in VHL negative and VHL positive tumours, the major clear cell renal cell carcinoma LOH region mapped to 3p21.3, close to the lung cancer tumour suppressor gene region 1 (LCTSGR1). There was no association between tumour VHL status and tumour grade and stage.

Conclusions—These findings further indicate that VHL inactivation is not sufficient to initiate clear cell renal cell carcinoma and that loss of a gatekeeper 3p21 tumour suppressor gene is a crucial event for renal cell carcinoma development in both VHL negative and VHL positive clear cell renal cell carcinoma.

(J Clin Pathol: Mol Pathol 2000;53:137–144)

Keywords: VHL; chromosome 3p; loss of heterozygosity; renal cell carcinoma

Renal cell carcinoma accounts for approximately 2% of adult malignancies and is the most common adult kidney neoplasm. Renal cell carcinoma is histopathologically heterogeneous, most sporadic renal cell carcinomas (about 80%) are classified as clear cell (or non-papillary) tumours, with non-clear cell tumours predominantly classed as chromophilic (12%) or chromophobe (5%). Cytogenetic and molecular genetic studies in the 1980s identified chromosome 3p deletions as the most common genetic event in renal cell carcinoma,^{1–3} and subsequently the VHL tumour suppressor gene was mapped to chromosome 3p25.^{4–6} Germline mutations of the VHL tumour suppressor gene cause von Hippel-Lindau (VHL) disease and are associated with a high risk of early onset and multicentric clear cell renal cell carcinoma.^{7–9} Somatic VHL gene mutations are found in 43–57% of clear cell renal cell carcinoma cell lines and primary tumours,^{10–11} and an additional 11–19% of renal cell carcinoma cell lines and primary tumours demonstrate promoter hypermethylation and transcriptional silencing of the VHL gene.^{12–14} VHL gene inactivation is not a feature of non-clear cell renal cell carcinoma, and recently germline and somatic mutations in the MET protooncogene have been reported in familial and sporadic non-clear cell papillary renal cell carcinoma.^{15–16} The reintroduction of wild-type VHL into VHL null clear cell renal cell carcinoma cell lines suppresses tumorigenicity in vivo.¹⁷ These studies clearly implicate inactivation of VHL in the pathogenesis of many clear cell renal cell carcinomas and establish VHL as a crucial gatekeeper for the most common form of kidney cancer. However, it is likely that other clear cell renal cell carcinoma gatekeeper genes remain to be identified.^{14–18} Thus, a considerable proportion of sporadic clear cell renal cell carcinomas do not have evidence of VHL inactivation and hereditary clear cell carcinoma kindreds are not allelic with VHL disease. In a previous smaller study, we identified frequent chromosome 3p allele loss in sporadic clear cell renal cell carcinoma, with and without VHL inactivation, suggesting that additional non-VHL chromosome 3p tumour suppressor

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Accepted for publication
21 March 2000

genes play a crucial role in the pathogenesis of clear cell renal cell carcinoma.¹⁴ This hypothesis is consistent with reports that, in chromosome transfer experiments, fragments of chromosome 3p regions not containing the VHL gene (notably, 3p12–13, 3p14.2, and 3p21–22 regions) can suppress renal cell carcinoma tumorigenicity.^{19–20}

Multiple lines of evidence suggest the existence of several 3p tumour suppressor genes in addition to VHL. The finding of overlapping homozygous deletions in lung and breast tumours and tumour cell lines at 3p21.3^{21–24} and 3p12^{25–26} is important evidence for tumour suppressor genes at these regions (lung cancer tumour suppressor gene region 1 (LCTSGR1) and LCTSGR2, respectively). LCTSGR1 is cloned in a cosmid contig of 700 kb containing the overlap of 120 kb of homozygous deletions in one breast and three lung tumour cell lines.²⁴ LCTSGR2 is cloned in a yeast artificial chromosome contig of 8 Mb, containing overlapping homozygous deletions in one breast and two lung tumour cell lines.²⁶ Interestingly, the 3p12 region implicated in renal cell carcinoma by microcell mediated tumour suppression studies overlaps the LCTSGR2 region.^{27–28} In addition to the as yet unidentified tumour suppressor genes at 3p12 and 3p21, the FHIT gene at 3p14.2 has been proposed as a tumour suppressor gene in many common cancers. FHIT is a large gene covering > 1000 kb of genomic DNA; it encompasses the t(3:8) breakpoint region associated with familial clear cell renal cell carcinoma, and the FRA3B fragile site. Homozygous deletions within the FHIT gene in breast, kidney, lung,^{29–34} and other cancers have been reported. Although kidney cancer also demonstrates frequent LOH at 3p14, the role of the FHIT gene in tumorigenesis is controversial, and it has been suggested that frequent LOH at 3p14 might reflect the presence of the fragile site (and hence susceptibility to rearrangements) in this region.

The major objective of our present study was to investigate further the role of 3p tumour suppressor genes in renal cell carcinoma by analysing in detail LOH at three candidate renal cell carcinoma tumour suppressor gene regions—LCTSGR1, LCTSGR2, and 3p14—in a large series of well characterised clear cell renal cell carcinomas with and without VHL inactivation.

Materials and methods

PATIENTS AND TUMOUR SPECIMENS

A total of 82 primary human renal cell carcinomas and matched normal kidney tissues were collected at Yokohama City University Hospital and its affiliated hospitals. All tumours were classified according to Thoenes' pathological typing of human renal cell carcinoma.³⁵ Clear cell renal cell carcinoma tumours were classified as: stage 1 (five), stage 2 (38), stage 3 (24), and stage 4 (five); grade 1 (21), grade 2 (42), and grade 3 (11). VHL mutational analysis has been described previously.³⁶ VHL methylation analysis was done using the methylation specific polymerase

Figure 1 Summary of polymorphic markers and regions of interest on chromosome region 3p. Genetic markers are listed in descending order from telomere to centromere according to published maps and approximate cytogenetic positions. The regions implicated in lung cancer development at 3p21.3 and 3p12 (LCTSGR1 and LCTSGR2) are also shown, as are the positions of the VHL and FHIT genes.

chain reaction (PCR) method and primers described by Herman *et al.*³⁷

LOSS OF HETEROZYGOSITY (LOH) ANALYSIS

High molecular weight genomic DNAs were extracted from tumours and normal kidney tissue by standard procedures.

PCR amplification of dinucleotide and trinucleotide microsatellite sequences was performed along with PCR based restriction fragment length polymorphism (RFLP). Eleven markers were selected spanning the regions of interest on 3p. All are available through Genome Data Bases (GDB at <http://gdbwww.gdb.org/>), with the exception of new primers for the D3S1621 locus (forward primer, 5'-CCTCACTACTCCTGGAATTG-3'; reverse primer, 5'-CCAAGGAAGGGTTTACTTA-3'; PCR product size 140 bp, annealing temperature 55°C).

Figure 2 Chromosome 3p allelotyping analysis of (A) 41 clear cell renal cell carcinomas with *VHL* mutation or methylation (*VHL* negative) and (B) 41 clear cell renal cell carcinomas without *VHL* inactivation (*VHL* positive) using 11 microsatellite markers. Case numbers are shown on the top. Filled circles, loss of heterozygosity; open circles, retention of heterozygosity; no symbol: not informative.

The forward primers were end radiolabelled in a 10 µl reaction volume containing 2 pmol of primer for each reaction, 1× T4 PNK buffer (MBI Fermentas, Sunderland, Tyne and Wear, UK); 10 U of T4 polynucleotide kinase (MBI Fermentas), and 30 µCi [γ^{32} P]dATP (3000 Ci/mmol) (Amersham Life Science, Amersham, Buckinghamshire, UK). This reaction was then incubated at 37°C for 30 minutes, and 94°C for 5 minutes. The PCR was performed in a volume of 10 µl containing 100 ng of genomic DNA; 1× PCR buffer (Gibco-BRL Paisley, UK); 3 MgCl₂; 100 µM each of dTTP, dGTP, dCTP, and dATP; 0.05% W-1; 0.5 U Taq polymerase (Life Technologies Ltd, Gibco,

Paisley, UK). Amplification was carried out with initial denaturation at 94°C for five minutes, followed by 30 cycles consisting of denaturation at 94°C for 30 seconds, annealing primer specific temperature for 30 seconds, and elongation at 72°C for 30 seconds. The final extension was for 10 minutes at 72°C. The PCR products were mixed in a ratio 1 : 1 with a solution of 95% formamide, 10 mM EDTA, 10% bromophenol blue, and 10% xylene cyanol. Denaturation was at 95°C for five minutes and the product was loaded on to a 6% polyacrylamide denaturing gel (Sequagel-6; National Diagnostics Hull, UK). Electrophoresis was carried out for two to four

Figure 3 Representative autoradiographs of microsatellite analysis for loss of heterozygosity (LOH) at 3p21 loci in (A) VHL negative and (B) VHL positive clear cell renal cell carcinoma. N and T indicate matched DNA samples isolated from normal and tumour tissues, respectively. The case number is at the bottom and the 3p locus at the top of each autoradiograph. Arrows indicate the alleles, bold arrows indicate the lost allele in the tumour.

hours at 90 W constant power to achieve adequate separation of alleles. After drying, the gel was exposed to x ray film (Fuji, Tokyo, Japan).

STATISTICAL ANALYSIS
Comparisons were made by Fisher’s exact test.

Results
Eighty two matched clear cell renal cell carcinoma tumour/normal DNA pairs were analysed for LOH at 11 polymorphic loci on chromosome 3p. Forty one tumours had VHL inactivation (mutations or methylation) (VHL negative tumours) and 41 had no VHL tumour suppressor gene inactivation (VHL positive tumours). The 11 polymorphic markers were targeted to include the following 3p tumour suppressor gene candidate regions: 3p25 (D3S1317 and D3S1038); 3p21–22 (UBE1L, D3S2407, D3S1568, D3S1621, and D3S2408); 3p14 (D3S1481); and 3p12 (D3S3507, D3S1274, and D3S1604). The 3p25 markers used are just proximal to the VHL tumour suppressor gene locus, the 3p21.3 markers contain region LCTSGR1, the marker at 3p14.2 is within the FHIT locus, and three markers at 3p12 represent region LCTSGR2 (fig 1).

All tumours were informative for at least one marker, and LOH at one or more locus was found in 65 of 82 (79%) of the clear cell renal cell carcinomas analysed in our study. Fifteen tumours showed loss for every informative marker and 17 tumours showed no loss for any informative loci.

CHROMOSOME 3p LOH IN CLEAR CELL RENAL CELL CARCINOMA WITH AND WITHOUT VHL INACTIVATION

Figure 2 gives detailed LOH data for all tumours with VHL inactivation (mutation or methylation) and without VHL inactivation, and fig 3 shows representative examples of LOH at 3p21 loci. A comparison of LOH frequencies at 3p25 and 3p12–p21 in all informative VHL negative and VHL positive tumours showed that the frequency of LOH at 3p25 and at 3p12–p22 was similar in VHL negative tumours (30 of 39 (78%) *v* 37 of 41 (90%); *p* = 0.14). In contrast, the frequency of LOH at 3p25 was significantly less than that at 3p12–p22 in VHL positive tumours (17 of 38 (45%) *v* 28 of 41 (68%); *p* = 0.0426). We also compared those VHL positive tumours with LOH in at least one locus to VHL negative tumours (table 1) in case normal DNA contamination was preventing us from picking up LOH in tumour DNA. The frequency of 3p12–p22 LOH was similar in both tumour sets: VHL negative, 37 of 41 (90%) and VHL positive with LOH, 28 of 28 (100%); *p* = 0.14. However, all of the VHL negative tumours that demonstrated 3p25 LOH also demonstrated 3p12–p22 LOH (for example, tumours 62, 71, and 79 in figure 2A), and the frequency of LOH at 3p25 and at 3p12–p22 was similar in VHL negative tumours (30 of 39 (78%) *v* 37 of 41 (90%); *p* = 0.14). In contrast, the frequency of LOH at 3p25 was significantly less than that at 3p12 p22 (17 of 25 (68%) *v* 28 of 28 (100%); *p* = 0.001) in VHL positive tumours

Table 1 Comparison of loss of heterozygosity (LOH) frequencies between different regions of 3p in tumours with and without VHL inactivation

% LOH frequencies					Significance of difference between 3p12–22 and 3p25
3p25	3p21–22	3p14	3p12	3p12–22	
VHL inactivation (VHL [−])					
78 (30/39)	85 (35/41)	50 (8/16)	62 (23/37)	90 (37/41)	p = 0.14
3p LOH with no VHL inactivation (VHL ⁺ /LOH)					
68 (17/25)	89 (25/28)	55 (6/11)	52 (13/25)	100 (28/28)	p = 0.0012
Significance of difference for each region					
p = 0.560	p = 0.43	p = 1.0	p = 0.45	p = 0.14	

VHL inactivation is via methylation or mutation.
The significance of the difference between 3p12–22 and 3p25 was measured using Fisher’s exact test.

Figure 4 Comparison of tumour stage and grade distributions in clear cell renal cell carcinoma with VHL inactivation (VHL⁻), clear cell renal cell carcinoma without VHL inactivation (VHL⁺), and clear cell renal cell carcinoma without VHL inactivation but loss of at least one informative 3p marker (VHL⁺/LOH)

with LOH. In addition, there were no VHL positive tumours that showed LOH at 3p25 but not 3p12–p22. These observations suggested the following: (1) VHL inactivation without loss of 3p12–p22 tumour suppressor gene(s) is not sufficient for renal cell carcinoma development, and (2) 3p12–p22 tumour suppressor gene(s) are implicated in both VHL dependent and VHL independent pathways of clear cell renal cell carcinoma development.

We then proceeded to compare the patterns of 3p LOH in VHL negative and VHL positive clear cell renal cell carcinoma to determine which of the candidate tumour suppressor gene regions were most likely to be involved in the two subgroups. For VHL negative tumours, 20 of 41 tumours showed complete 3p LOH (for example, tumours 288, 164, 177, 105, 111, 53, 70, 212, 224, 135, 138, and 286) or a pattern of distal loss with retention of proximal markers (for example, tumours 62, 71, 129, 258, etc) (fig 2A). The analysis of the tumours with a telomeric LOH pattern clearly suggested that a loss of 3p21 tumour suppressor gene(s) was necessary for renal tumorigenesis in these VHL negative tumours and that the 3p21 tumour

suppressor gene mapped distal to D3S2408, which includes the LCTSGR1. For those VHL negative clear cell renal cell carcinomas with more complex interstitial LOH patterns, there was evidence that 3p12 or 3p14 LOH was involved in some cases (for example, tumours 45, 243, and 65) (fig 2A); however, in each case there was also LOH at 3p21, or 3p21 markers were uninformative (for example, tumour 45), such that there was no unequivocal evidence that inactivation of the 3p12–p14 tumour suppressor genes can substitute for inactivation of a 3p21 tumour suppressor gene in VHL negative tumours.

For VHL positive tumours, 17 of 41 demonstrated complete 3p LOH (for example, tumours 306, 192, and 82) or a pattern of distal loss with retention of proximal markers (for example, tumours 63, 69, and 107) (fig 2B). The proximal breakpoint in the tumours was similar to that seen in VHL negative tumours with similar patterns of LOH, although the 3p21 renal cell carcinoma tumour suppressor gene candidate region could be mapped slightly more telomeric (that is, distal to D3S1621). Again this placed LCTSGR1 within the putative 3p21 clear cell renal cell carcinoma tumour suppressor gene candidate region. The analysis of VHL positive tumours with interstitial patterns of 3p LOH demonstrated that in most cases 3p21 was included in the region of LOH, and suggested that the 3p21 clear cell renal cell carcinoma tumour suppressor gene crucial region is in the D3S1621 to UBE1L interval (which includes LCTSGR1). However, in addition to 3p21 LOH, several VHL positive tumours also demonstrated interstitial deletions involving 3p12 or 3p14, and two (tumours 2 and 285) demonstrated LOH at 3p14 only (although the LOH could extend into 3p12 in 285).

Interstitial 3p12 LOH in the presence of 3p21 LOH was relatively common in VHL positive and VHL negative clear cell renal cell carcinoma (tumours 243, 160, 93, 103, etc), but the only tumour with apparent 3p12 LOH only was not informative for all 3p21 markers (tumour 259). Analysis of those tumours with 3p12 LOH in the presence of 3p21 LOH suggested that the most likely region for a 3p12 clear cell renal cell carcinoma tumour suppressor gene was centromeric to D3S3507, within the LCTSGR2 interval.

COMPARISON OF TUMOUR GRADE AND STAGE IN VHL NEGATIVE AND VHL POSITIVE CLEAR CELL RENAL CELL CARCINOMA

Because the stage and grade of most clear cell renal cell carcinomas (75 of 82 tumours with known stage and 76 of 82 with known grade) in our study were known, we investigated whether different mechanisms of clear cell renal cell carcinoma tumorigenesis (VHL dependent or VHL independent) were associated with differences in tumour stage and grade. The stage and grade distributions for (1) clear cell renal cell carcinoma with VHL inactivation, (2) clear cell renal cell carcinoma with no evidence of VHL inactivation, and (3) clear cell renal cell carcinoma with no evidence of inactivation but

with LOH in at least one 3p locus were compared (fig 4). There were no significant differences in stage or grade frequencies between the three groups.

Chromosome 3p LOH appeared to be an early event in clear cell renal cell carcinoma; thus, four of five stage 1 tumours demonstrated LOH in at least one 3p marker, compared with five of five stage 4 tumours. There were no clear differences in the pattern of 3p LOH between stage 1 and stage 4 tumours.

Discussion

To compare the mechanisms of tumorigenesis in clear cell renal cell carcinoma with and without VHL gene inactivation, we analysed the pattern and extent of allelic losses on 3p in a large set of clear cell renal cell carcinomas of known VHL status. We have shown that: (1) 3p loss occurs in a high proportion of clear cell renal cell carcinomas (79% LOH for one or more 3p markers); (2) 3p12–p22 LOH occurs irrespective of VHL inactivation status; (3) no VHL negative or VHL positive clear cell renal cell carcinomas demonstrated only 3p25 LOH; (4) although the frequency of 3p25 and 3p12–p22 LOH was similar in VHL negative clear cell renal cell carcinoma, in VHL positive tumours with LOH, 3p25 LOH was less common than 3p12–p22 LOH; and (5) although there was evidence for multiple clear cell renal cell carcinoma tumour suppressor genes in 3p12–p22, the major one mapped to 3p21.3, close to the LCTSGR1. Our results provide intriguing insights into the mechanisms of clear cell renal cell carcinoma tumorigenesis. Most clear cell renal cell carcinomas have evidence of VHL gene inactivation and VHL has a gatekeeper role for clear cell renal cell carcinoma. Although the precise functions of the VHL gene product are still under investigation, recent studies suggest that a major role of the VHL product is to regulate degradation by the proteasome of target proteins such as HIF-1.³⁸ Thus, the multimeric complex formed by the VHL protein, elongin B and C proteins, CUL2, NEDD8, and RBx1, has close similarities to the SCF complex known to be involved in ubiquitin mediated proteolysis in yeast.^{39–44} VHL negative clear cell renal cell carcinoma cell lines constitutively overexpress the gene encoding vascular endothelial factor (VEGF) and other hypoxia inducible genes,^{45–47} and this correlates with abnormal stabilisation of HIF-1 and HIF-2 transcription factors in VHL negative cells.³⁸ The upregulation of VEGF mRNA expression by HIF-1 is consistent with the highly vascular nature of many clear cell renal cell carcinomas. In addition to regulating tumour angiogenesis, the VHL protein has also been reported to influence the tumour expression of matrix metalloproteases and their inhibitors (for example, MMb-9 and TIMP-2; JR Gnarr, personal communication, 1998) and to modulate extracellular fibronectin deposition.⁴⁸ Thus, loss of VHL protein function in clear cell renal cell carcinoma cells might influence tumorigenesis by a variety of mechanisms.

Although VHL gene inactivation is clearly important for clear cell renal cell carcinoma tumorigenesis, our chromosome 3p LOH studies in VHL negative tumours suggest that VHL inactivation is not sufficient for tumorigenesis, and that the inactivation of additional chromosome 3p tumour suppressor gene(s) is also required. This is consistent with the findings of our previous smaller study, which did not use 3p markers targeted to candidate 3p tumour suppressor gene regions. In our current study, we used polymorphic markers at key candidate 3p tumour suppressor gene regions, and it was of considerable interest to note the similarities in 3p LOH loss at 3p12–p22, both in VHL negative and VHL positive tumours with LOH. Our data suggest that the VHL dependent and VHL independent pathways of clear cell renal cell carcinoma tumorigenesis converge on inactivation of a 3p21 tumour suppressor gene. Thus, although several candidate 3p12–p22 tumour suppressor genes have been implicated in the pathogenesis of several common cancers, our results strongly suggest that the major clear cell renal cell carcinoma tumour suppressor gene maps to 3p21. LOH at 3p14 and 3p12 was a common finding in VHL negative and VHL positive clear cell renal cell carcinoma, but was usually observed in the presence of 3p21 LOH. The FHIT gene at 3p14.2 has been implicated in clear cell renal cell carcinoma tumorigenesis by some groups (but not others), and although our results do not exclude a role for FHIT and a 3p12 tumour suppressor gene, they do suggest that these are of lesser importance than the 3p21 tumour suppressor gene. The 3p21 clear cell renal cell carcinoma tumour suppressor gene candidate region contains LCTSGR1, which is defined by homozygous deletions both in lung and breast cancer and is covered by a 700 kb contig.²³ Several candidate genes have been isolated from LCTSGR1,^{49–51} but to date no mutations have been reported in lung cancer. These genes have not been analysed in clear cell renal cell carcinoma, so it is unclear whether the 3p21 lung cancer and clear cell renal cell carcinoma tumour suppressor genes will be identical or merely colocalise to the same region. The mapping of proximal 3p LOH in VHL negative and VHL positive tumours localised a 3p12 clear cell renal cell carcinoma tumour suppressor gene region to the LCTSGR2 interval. Recently, functional evidence has shown that LCTSGR2 is also important in renal cell carcinoma development,^{27, 28} and the identification and mutation analysis of LCTSGR2 candidate genes will elucidate whether lung cancer and clear cell renal cell carcinoma tumour suppressor genes are allelic.

We did not identify any differences in tumour grade and stage between VHL negative and VHL positive clear cell renal cell carcinomas. These observations are consistent with the similarity in 3p12–p22 LOH analysis in the two groups. However, it is unclear whether VHL gene inactivation would be expected to influence tumour biology (as assessed by tumour stage and grade). The absence of

differences between VHL negative and VHL positive clear cell renal cell carcinoma could indicate that VHL inactivation did not influence the tumour stage or grade or that VHL positive tumours with LOH have an equivalent inactivation event elsewhere in the VHL pathway. Thus, the inactivation of another protein in the VHL, elongin B and C, CUL2, NEDD8, RBx1 complex might be functionally equivalent to VHL inactivation. However, to date, mutations in CUL2 have not been identified in sporadic VHL positive clear cell renal cell carcinoma or in familial non-VHL clear cell renal cell carcinoma.⁵² Mutation analysis of the genes encoding the other VHL interacting proteins in sporadic and familial clear cell renal cell carcinoma will determine their role in the pathogenesis of VHL positive tumours.

This is the largest study to date comparing the pattern of LOH in clear cell renal cell carcinoma with and without known VHL mutation or methylation. We have confirmed and greatly extended previous studies indicating the presence of tumour suppressor genes on 3p other than VHL that are important in renal cell carcinoma development. Furthermore, we demonstrate that the LCTSGR1 and LCTSGR2 regions, which are implicated in the development of other common sporadic cancers, such as lung and breast, also play a major role in renal cell carcinoma tumour development. In addition, we mapped two small non-overlapping deletions in clear cell renal cell carcinoma (at 3p21 and 3p12). These will help in isolating candidate tumour suppressor genes and determining the extent of their involvement in renal cell carcinoma development.

A Martinez was supported by the University of Antioquia, Medellin, Colombia, and a fellowship from COLCIENCIAS.

- Zbar B, Brauch H, Talmadge C, *et al.* Loss of alleles of loci on the short arm of chromosome-3 in renal-cell carcinoma. *Nature* 1987;327:721-4.
- Kovacs G, Erlandsson R, Boldog F, *et al.* Consistent chromosome 3p deletion and loss of heterozygosity in renal cell carcinoma. *Proc Natl Acad Sci U S A* 1988;85:1571-5.
- van der Hout AH, Kok K, van den Berg A, *et al.* Direct molecular analysis of a deletion of 3p in tumours from patients with sporadic renal cell carcinoma. *Cancer Genet Cytogenet* 1988;32:281-5.
- Hosoe S, Brauch H, Latif F, *et al.* Localization of the von Hippel-Lindau disease gene to a small region of chromosome 3. *Genomics* 1990;8:634-40.
- Maher ER, Bently E, Yates JRW, *et al.* Mapping of the von Hippel-Lindau disease locus to a small region of chromosome 3p by genetic linkage analysis. *Genomics* 1991;10:957-60.
- Latif F, Tory K, Gnarr J, *et al.* Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 1993;260:1317-20.
- Maher ER, Yates JR, Harries R, *et al.* Clinical features and natural history of von Hippel-Lindau disease. *Q J Med* 1990;77:1151-63.
- Zbar B. Von Hippel-Lindau disease and sporadic renal cell carcinoma. *Cancer Surv* 1995;25:219-32.
- Maher ER, Kaelin WG. Von Hippel-Lindau disease. *Medicine* 1997;76:381-91.
- Gnarr JR, Tory K, Weng Y, *et al.* Mutation of the VHL tumour suppressor gene in renal carcinoma. *Nat Genet* 1994;7:85-90.
- Foster K, Prowse A, van den Berg A, *et al.* Somatic mutations of the von Hippel-Lindau disease tumour suppressor gene in nonfamilial clear cell renal carcinoma. *Hum Mol Genet* 1994;3:2169-73.
- Herman JH, Latif F, Weng Y, *et al.* Silencing of the VHL tumour suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci U S A* 1994;91:9700-4.
- Prowse AH, Webster AR, Richards FM, *et al.* Somatic inactivation of the VHL gene in von Hippel-Lindau disease tumors. *Am J Hum Genet* 1997;60:765-71.
- Clifford SC, Prowse AH, Affara NA, *et al.* Inactivation of the von Hippel-Lindau (VHL) tumour suppressor gene and allelic losses at chromosome arm 3p in primary renal cell carcinoma: evidence for a VHL-independent pathway in clear cell renal tumorigenesis. *Genes Chromosomes Cancer* 1998;22:200-9.
- Schmidt L, Duh FM, Chen F, *et al.* Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. *Nat Genet* 1997;16:68-73.
- Schmidt L, Junker K, Nakaigawa N, *et al.* Novel mutations of the MET proto-oncogene in papillary renal carcinomas. *Oncogene* 1999;18:2343-50.
- Iliopoulos O, Kibel A, Gray S, *et al.* Tumour suppression by the human von Hippel-Lindau gene product. *Nat Med* 1995;1:822-6.
- Van den Berg A, Hulsbeek MF, de Jong D, *et al.* Major role for a 3p21 region and lack of involvement of the t(3;8) breakpoint region in the development of renal cell carcinoma suggested by loss of heterozygosity analysis. *Genes Chromosomes Cancer* 1996;15:64-72.
- Killary AM, Wolf ME, Giambernardi TA, *et al.* Definition of a tumor suppressor locus within human chromosome 3p21-p22. *Proc Natl Acad Sci U S A* 1992;89:10877-81.
- Sanchez Y, Elnaggar A, Pathak S, *et al.* A tumor suppressor locus within 3p14-p12 mediates rapid cell death of renal cell carcinoma in vivo. *Proc Natl Acad Sci U S A* 1994;91:3383-7.
- Daly MC, Xiang RH, Buchhagen D, *et al.* A homozygous deletion on chromosome 3 in a small cell lung cancer cell line correlates with a region of tumor suppressor activity. *Oncogene* 1993;8:1721-9.
- Kok K, van den Berg A, Veldhuis PM, *et al.* A homozygous deletion in a small cell lung cancer cell line involving a 3p21 region with a marked instability in yeast artificial chromosomes. *Cancer Res* 1994;54:4183-7.
- Wei MH, Latif F, Bader S, *et al.* Construction of a 600-kilobase cosmid clone contig and generation of a transcriptional map surrounding the lung cancer tumor suppressor gene (TSG) locus on human chromosome 3p21.3: progress toward the isolation of a lung cancer TSG. *Cancer Res* 1996;56:1487-92.
- Sekido Y, Mohsen A, Wistuba II, *et al.* Cloning of a breast cancer homozygous deletion junction narrows the region of search for a 3p21.3 tumor suppressor gene. *Oncogene* 1998;16:3151-7.
- Latif F, Tory K, Modi W, *et al.* Molecular characterization of a large homozygous deletion in the small cell lung cancer line U2020: a strategy for cloning the putative tumor suppressor gene. *Genes Chromosomes Cancer* 1992;5:119-27.
- Sundaresan V, Chung G, Heppell-Parton A, *et al.* Homozygous deletions at 3p12 in breast and lung cancer. *Oncogene* 1998;17:1723-9.
- Lott ST, Lovell M, Naylor SL, *et al.* Physical and functional mapping of a tumor suppressor locus for renal cell carcinoma within chromosome 3p12. *Cancer Res* 1998;58:3533-7.
- Lovell M, Lott ST, Wong P, *et al.* The genetic locus NRC-1 within chromosome 3p12 mediates tumor suppression in renal cell carcinoma independently of histological type, tumor microenvironment, and VHL mutation. *Cancer Res* 1999;59:2182-9.
- Negrini M, Monaco C, Vorechovsky I, *et al.* The FHIT gene at 3p14.2 is abnormal in breast carcinomas. *Cancer Res* 1996;56:3173-9.
- Ohta M, Inoue H, Cotticelli MG, *et al.* The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. *Cell* 1996;84:587-97.
- Sozzi G, Veronese ML, Negrini M, *et al.* The FHIT gene 3p14.2 is abnormal in lung cancer. *Cell* 1996;85:17-26.
- Druck T, Hadaczek P, Fu TB, *et al.* Structure and expression of the human FHIT gene in normal and tumor cells. *Cancer Res* 1997;57:504-12.
- Fong KM, Biesterveld EJ, Virmani A, *et al.* FHIT and FRA3B 3p14.2 allele loss are common in lung cancer and preneoplastic bronchial lesions and are associated with cancer-related FHIT cDNA splicing aberrations. *Cancer Res* 1997;57:2256-67.
- Huebner K, Hadaczek P, Siprashvili Z, *et al.* The FHIT gene, a multiple tumor suppressor gene encompassing the carcinogen sensitive chromosome fragile site, FRA3B. *Biochim Biophys Acta* 1997;1332:M65-70.
- Thoenes W, Storkel S, Rumpelt HJ. Histopathology and classification of renal cell tumors (adenomas, oncocytomas and carcinomas). The basic cytological and histopathological elements and their use for diagnostics. *Pathol Res Pract* 1986;181:125-43.
- Shuin T, Kondo K, Torigoe S, *et al.* Frequent somatic mutations and loss of heterozygosity of the von Hippel-Lindau tumor suppressor gene in primary human renal cell carcinomas. *Cancer Res* 1994;54:2852-5.
- Herman JG, Graff JR, Myohanen S, *et al.* Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* 1996;93:9821-6.
- Maxwell PH, Wiesener MS, Chang GW, *et al.* The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 1999;399:271-5.
- Duan DR, Pause A, Burgess WH, *et al.* Inhibition of transcription elongation by the VHL tumor suppressor protein. *Science* 1995;269:1402-6.

- 40 Kibel A, Iliopoulos O, DeCaprio JA, *et al.* Binding of the von Hippel-Lindau tumor suppressor protein to elongin B and C. *Science* 1995;**269**:1444–6.
- 41 Pause A, Lee S, Worrell RA, *et al.* The von Hippel-Lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins. *Proc Natl Acad Sci U S A* 1997;**94**: 2156–61.
- 42 Lonergan KM, Iliopoulos O, Ohh M, *et al.* Regulation of hypoxia-inducible mRNAs by the von Hippel-Lindau tumor suppressor protein requires binding to complexes containing elongins B/C and Cul2. *Mol Cell Biol* 1998;**18**: 732–41.
- 43 Kamura T, Koepp DM, Conrad MN, *et al.* Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. *Science* 1999;**284**:657–61.
- 44 Liakopoulos D, Busgen T, Brychzy A, *et al.* Conjugation of the ubiquitin-like protein NEDD8 to cullin-2 is linked to von Hippel-Lindau tumor suppressor function. *Proc Natl Acad Sci U S A* 1999;**96**:5510–15.
- 45 Gnarr JR, Zhou S, Merrill MJ, *et al.* Post-transcriptional regulation of vascular endothelial growth factor mRNA by the product of the VHL tumor suppressor gene. *Proc Natl Acad Sci U S A* 1996;**93**:10589–94.
- 46 Iliopoulos O, Levy AP, Jiang C, *et al.* Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. *Proc Natl Acad Sci U S A* 1996;**93**:10595–9.
- 47 Siemeister G, Weindel K, Mohrs K, *et al.* Reversion of deregulated expression of vascular endothelial growth factor in human renal carcinoma cells by von Hippel-Lindau tumor suppressor protein. *Cancer Res* 1996;**56**: 2299–301.
- 48 Ohh M, Yauch RL, Lonergan KM, *et al.* The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. *Molecular Cell* 1998;**1**:959–68.
- 49 Sekido Y, Bader S, Latif F, *et al.* Human semaphorins A(V) and IV reside in the 3p21.3 small cell lung cancer deletion region and demonstrate distinct expression patterns. *Proc Natl Acad Sci U S A* 1996;**93**:4120–4125.
- 50 Sithanandam G, Latif F, Duh FM, *et al.* 3pK, a new mitogen-activated protein kinase-activated protein kinase located in the small cell lung cancer tumor suppressor gene region. *Mol Cell Biol* 1996;**16**:868–76.
- 51 Latif F, Duh FM, Bader S, *et al.* The human homolog of the rodent immediate early response genes, PC4 and TIS7, resides in the lung cancer tumor suppressor gene region on chromosome 3p21. *Hum Genet* 1997;**99**:334–41.
- 52 Clifford SC, Walsh S, Hewson K, *et al.* Genomic organization and chromosomal localization of the human CUL2 gene and the role of von Hippel-Lindau tumor suppressor-binding protein (CUL2 and VBP1) mutation and loss in renal-cell carcinoma development. *Genes Chromosomes Cancer* 1999;**26**:20–8.