Familial clear cell renal cell carcinoma (FCRC): clinical features and mutation analysis of the VHL, MET, and CUL2 candidate genes

Emma R Woodward, Steven C Clifford, Dewi Astuti, Nabeel A Affara, Eamonn R Maher

Abstract
Familial renal cell carcinoma (RCC) is genetically heterogeneous. Genetic predisposition to clear cell RCC (CCRCC) is a major feature of von Hippel-Lindau (VHL) disease (MIM 193300) and has rarely been associated with chromosome 3 translocations. In addition, familial papillary (non-clear cell) RCC may result from germline mutations in the MET proto-oncogene (MIM 164860). However, rare kindreds with familial CCRCC (FCRC) not linked to the VHL tumour suppressor gene have been described suggesting that further familial RCC susceptibility genes exist. To investigate the genetic epidemiology of FCRC, we undertook a clinical and molecular study of FCRC in nine kindreds with two or more cases of CCRCC in first degree relatives. FCRC was characterised by an earlier age at onset (mean 47.1 years, 52% of cases <50 years of age) than sporadic cases. These findings differ from the only previous report of two FCRC kindreds and have important implications for renal surveillance in FCRC. The molecular basis of CCRCC susceptibility was investigated in nine FCRC kindreds and seven isolated cases with features of possible genetic susceptibility to CCRCC (four bilateral CCRCC aged <50 years and three with unilateral CCRCC aged <30 years). No germline mutations were detected in the VHL or MET genes, suggesting that FCRC is not allelic with VHL disease or HPRC. As binding of the VHL gene product to the CUL2 protein is important for pVHL function, we then searched for germline CUL2 mutations. Although CUL2 polymorphisms were identified, no pathogenic mutations were detected. These findings further define the clinical features of FCRC and exclude a major role for mutations in VHL, MET, or CUL2 in this disorder.

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Keywords: familial clear cell renal carcinoma; VHL; MET; CUL2

Renal cell carcinoma (RCC) accounts for ~2% of all cancers in the western world. RCC is histologically heterogeneous, with most (~80%) classified as clear cell (CCRCC). Among the non-clear cell types, papillary (chromophilic) and chromophobic tumours are the most frequent.1 Only about 2% of all cases of RCC are familial,2 but identification of the genetic basis of RCC susceptibility has provided important insights into the molecular pathogenesis of RCC. The most common cause of familial clear cell RCC (FCRC) is VHL disease (MIM 193300), which is caused by germline mutation of the VHL tumour suppressor gene and is characterised by predisposition to retinal and cerebellar haemangioblastomas, CCRCC, and phaeochromocytoma.3,4 The lifetime risk of RCC in VHL disease is >70% by the age of 60 years,5 but not all germline VHL mutations are associated with a high risk of RCC. Specific missense mutations (for example, Tyr98His) may predispose to haemangioblastomas and phaeochromocytoma, but rarely RCC (VHL type 2A phenotype).6,7 In addition, approximately 50% of patients with isolated familial phaeochromocytoma have missense VHL gene mutations,8 suggesting that specific VHL gene mutations might produce variant phenotypes.

Familial RCC is uncommon and before 1991 there had been 23 reports of 105 patients with familial RCC.9 These cases were characterised by dominant inheritance, early age at onset, and bilateral tumours. Although familial CCRCC associated with chromosome 3 translocations was first described in 1979 (t(3;6) and t(3;8)),10–13 familial non-clear cell papillary RCC (HPRC) was not well defined until 1994.14 Subsequently, many cases of HPRC have been shown to have germline MET gene mutations.15,16 As most descriptions of familial RCC predate the identification of HPRC and, in retrospect, some early reports are suggestive of VHL disease, information on the genetics of familial CCRCC is limited. Recently, however, Teh et al17 described two large kindreds with FCRC in which linkage to VHL was excluded. A notable feature of these two kindreds was that, unlike CCRCC in VHL disease, the diagnosis of RCC in these families was characterised by late onset (8/9 cases aged >50 years) and unilateral involvement. To define the clinical features and molecular basis of FCRC, we ascertained families with at least two first degree relatives with CCRCC and investigated candidate RCC susceptibility genes in FCRC kindreds and sporadic cases with features suggestive of possible cancer susceptibility (young age at onset and multicentric tumours).

Methods

PATIENTS
Families were referred by clinical geneticists and urologists. When possible, all cancer diagnoses were confirmed by tracing histopatho-
logical records and clinical case notes. Families with evidence of VHL disease were excluded from this study. Peripheral blood for DNA analysis was collected from (1) probands from nine kindreds with FCRC (defined as at least two cases of CCRCC in first degree relatives), (2) four patients with bilateral CCRCC aged <50 years, and (3) three cases of early onset (<30 years) CCRCC. All subjects had normal cytogenetic analysis. For comparison of the age at onset of RCC in FCRC cases and sporadic and VHL cases we used data from Maher et al.19 This historical series was used because currently most VHL patients have RCC diagnosed presymptomatically as a result of screening. Such cases would not be comparable to FCRC cases, as no RCC diagnoses in the RCC kindreds were made presymptomatically.

MOLECULAR GENETIC ANALYSIS

VHL gene
Mutation analysis was performed as described previously.19 In brief, this consisted of Southern analysis, SSCP, analysis and direct sequencing of the VHL gene coding sequence. This was estimated to detect ~83% of germline VHL gene mutations.19

MET gene
Sequence analysis of exons 16, 17, 18, and 19 of the MET proto-oncogene was performed using PCR based single strand conformation polymorphism (SSCP) and direct DNA sequencing methods, as previously described by Schmidt et al.19 20 A positive control germine MET mutation from a patient with HPRC was kindly provided by Laura Schmidt (Laboratory of Immunobiology, NCI-FCRF, Frederick, USA). PCR conditions and primer sequences are available from the authors.

CUL2 gene
The entire coding sequence of the CUL2 gene (exons 2 to 21) was screened for sequence variations as described previously.20 PCR conditions and primer sequences are available on our web site (http://www.bham.ac.uk/ICl/ CUL2.htm). Sequence variations detected by SSCP were analysed further by direct sequence analysis of PCR products using the d-rhodamine sequencing kit protocol and the ABI 377 DNA sequencer (both Applied Biosystems).

CHROMOSOME 3P AND 7q LINKAGE STUDIES
Microsatellite analysis was performed using a selection of markers from chromosome 3p25-26 (D3S1304, D3S1537, D3S1317, and D3S1038), 3p21-24 (D3S1259, D3S2431, D3S2432, D3S2407,UBE1L, D3S2968, and D3S2407), and 3p14 (D3S1300 and D3S1481). UBE1L is a polymorphic 9 bp deletion at 3p21.2-21.3. Markers were amplified in a 15 µl PCR mix containing 1.5 µl 10× PCR buffer, 1.5 µl low C dNTPs (2.5 mmol/l dATP, 2.5 mmol/l dGTP, 2.5 mmol/l dTTP, 0.42 mmol/l dCTP), 60 ng each primer, 0.3 U Taq polymerase, 100 ng DNA template, 9.46 µl H2O, and 0.04 µl α-32P dCTP (10 μCi/µl). Reactions were overlaid with 30 µl mineral oil and amplified in a thermal cycler (Perkin Elmer Cetus) with the following cycling profile: 94°C for three minutes followed by 25 cycles of 94°C for one minute, 55°C for one minute, 72°C for one minute. Seven µl of formamide loading dye was then added to each reaction and the samples stored at −20°C. The products underwent electrophoresis in 6% denaturing gels at 30-33 W for three to seven hours and were visualised by autoradiography.

Results

CLINICAL FEATURES OF FCRC
In total, nine kindreds containing two or more cases of CCRCC in first degree relatives were ascertained. Details of the families are summarised in table 1. Two or more generations were affected in seven of nine kindreds and inheritance of FCRC was compatible with dominant transmission. Mean age at onset was 47.1 years in all familial cases, and 12 of 23 (52%) affected subjects developed RCC before the age of 50 years. We then compared the distribution of age at RCC diagnosis in FCRC cases with a previous series of VHL disease and sporadic cases (fig 1). Previously we had observed a clear difference between the age at onset of RCC in VHL disease (mean 44.8 years) and sporadic cases (mean 61.8 years).17 The mean age at diagnosis of CCRCC in FCRC kindreds (47.1 years, range 21-68 years) was intermediate between that seen in VHL disease and sporadic cases, but clearly younger than that of sporadic cases. Inspection of the age distribution of FCRC cases suggested a bimodal distribution, with many cases having an early age at onset similar to that seen in VHL disease (fig 1). Bilateral or multicentric disease was present in five of 23 affected subjects. Inheritance of FCRC in our kindreds was generally consistent with dominant transmission (with infrequent non-penetration) and there was a similar number of males and females affected (n=12 and 11, respectively) with no evidence of parental sex specific transmission effects.

Molecular genetic analysis of FCRC
VHL and MET gene analysis
A DNA sample for VHL gene mutation analysis was analysed in 16 probands (nine with familial FCRC, four with multicentric/bilateral CCRCC aged <50 years, and three with CCRCC aged <30 years). All cases had normal cytogenetic analysis and no patients had personal or family history features suggestive of

<table>
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<th>Kindred</th>
<th>No affected with VHL</th>
<th>Generations involved</th>
<th>Mean age</th>
<th>RCC aged &lt;50 y</th>
<th>RCC aged &gt;50 y</th>
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VHL disease. Southern analysis, SCCP, and direct sequencing were performed as described previously. However, no germline VHL mutations or sequence variants were identified.

Specific germline missense MET mutations cause HPRC. To investigate the possibility that HPRC and FCRC might be allelic we sequenced the HPRC associated regions (exons 16-19) of MET in nine cases with CCRCC susceptibility (six with familial FCRC (kindreds A, B, C, G, H, and I), one with multicentric/bilateral CCRCC aged <50 years, and two with CCRCC aged <30 years). No germline MET mutations or sequence variants were identified.

CHROMOSOME 3p AND 7q LINKAGE ANALYSIS

After finding no evidence for FCRC being allelic with VHL disease or HPRC, we considered other candidate genes for FCRC. To investigate the role of tumour suppressor genes on the short arm of chromosome 3 in FCRC, we performed linkage studies in the largest kindred (family E). Analysis with 13 microsatellite markers from chromosome 3p14-p26 was performed. The markers and order (and, when defined, location in cM from pter according to the Marshfield chromosome 3 sex averaged linkage map (http://www.gdb.org/gdb-bin/) were: D3S1304 (22.3 cM), D3S1537 (27.7 cM), D3S1317, D3S1038, D3S1259 (36.7 cM), D3S2431 (42.1 cM), D3S2432 (57.9 cM), D3S2407 (67.9 cM), UBE1L, D3S2968, D3S2408 (74.4 cM), D3S1300 (80.3 cM), and D3S1481. Linkage to FCRC was excluded (by absence of allele sharing between affected subjects) at D3S1537 (3p25), D3S1259 (3p24), D3S2432 (3p22-p24), D3S2407 (3p21), D3S2408 (3p14-p21), and D3S1300 (3p14) (fig 2). Linkage analysis to loci flanking the MET gene (D7S1837 and D7S496) was uninformative (results not shown).

MUTATION ANALYSIS OF THE CUL2 CANDIDATE TUMOUR SUPPRESSOR GENE IN FCRC

To investigate CUL2 as a candidate familial RCC gene we searched for germline mutations by SSCP analysis of all 20 exons encompassing the CUL2 coding region (exons 2-21) in nine cases (six with familial FCRC (kindreds A, B, C, G, H, and I), one with multicentric/bilateral CCRCC aged <50 years, and two with CCRCC aged <30 years). In the nine samples analysed, four silent polymorphisms were identified: two novel polymorphisms, G1265A in exon 12 and G2617A in the 3'UTR (exon 21), and two known polymorphisms G2057A (exon 19) and G2538A (exon 21, 3'UTR).20 21

Discussion

CLINICAL FEATURES OF FCRC

This study extends the definition of the clinical phenotype of FCRC initiated by Teh et al. In their report of two large families with FCRC, we identified four silent polymorphisms in the CUL2 gene. Further investigation of this gene in additional FCRC families is warranted to determine its role in the development of this disease.
A familial clear cell renal cell carcinoma (FCRC) patient has germline VHL gene mutations without clinical or radiological evidence of VHL disease, and a VHL gene mutation is inactivated in sporadic CCRCC irrespective of whether VHL inactivation is a rare limiting step in the pathogenesis of sporadic CCRCC, it is not sufficient and inactivation of a somatic VHL gene mutation in sporadic CCRCC has not been unequivocally implicated in sporadic CCRCC. It represented a plausible candidate for FCRC. However, we excluded linkage to various loci in 3p14-p25 in a single informative FCRC kindred. This finding is consistent with the two families reported by Teh et al., in whom karyotype analysis showed no abnormalities and linkage to both VHL and 3p14.2 was also excluded. At this stage genetic heterogeneity with linkage to chromosome 3p in some families cannot be excluded, but the available evidence is against 3p14-p25 being the site of a major FCRC locus.

The gatekeeper function of the VHL tumour suppressor gene in CCRCC is analogous to the
role of *APC* in colorectal cancer.35 36 Recent studies have suggested that the *VHL* gene product is part of a multimeric complex (including *CUL2*, elongins B and C, and *RBX1*) with homology to the SCF complex in yeast that target certain proteins (for example, HIF-1 and EPAS) for ubiquitin mediated proteolysis.36-41 The ability of pVHL to bind Cull2 and elongin C appears to be important for pVHL function.37 38 So mutations in genes encoding these proteins might be functionally equivalent to *VHL* gene mutations. However, we did not identify pathogenic CUL2 mutations in FCRC or in sporadic CCRCC tumours.20 Thus the molecular basis of FCRC remains to be defined. Genes encoding other pVHL interacting proteins represent plausible candidates for further evaluation. In addition, the chromosome 8 gene involved in the t(3;8) associated with CCRCC has been identified (TRC8) and shown to have some homology to the human patched gene (*PTC*).42 Although the CCRCC susceptibility effect of chromosome 3 translocations may relate to chromosome instability rather than disruption of specific genes,44 45 a putative TRC8 gene mutation was reported in one of 32 sporadic RCC studied and so the role of TRC8 in sporadic and familial RCC merits further evaluation. The ascertainment and investigation of additional FCRC kindreds will facilitate molecular genetic studies of CCRCC susceptibility and further define the clinical features of FCRC.

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