Abstract
Familial renal cell carcinoma (RCC) is genetically heterogeneous. Genetic predisposition to clear cell RCC (CCRC) 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 \textit{MET} proto-oncogene (MIM 164860). However, rare kindreds with familial CCRC (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 CCRC 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 CCRC susceptibility was investigated in nine FCRC kindreds and seven isolated cases with features of possible genetic susceptibility to CCRC (four bilateral CCRC aged <50 years and three with unilateral CCRC aged <30 years). No germline mutations were detected in the \textit{VHL} or \textit{MET} genes, suggesting that FCRC is not allelic with VHL disease or HPRC. As binding of the \textit{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 \textit{VHL}, \textit{MET}, or \textit{CUL2} in this disorder.

Keywords: familial clear cell renal carcinoma; \textit{VHL}; \textit{MET}; \textit{CUL2}

Renal cell carcinoma (RCC) accounts for \textasciitilde2\% of all cancers in the western world. RCC is histologically heterogeneous, with most (\textasciitilde80\%) classified as clear cell (CCRC). Among the non-clear cell types, papillary (chromophlic) and chromophobic tumours are the most frequent.\textsuperscript{1} Only about 2\% of all cases of RCC are familial,\textsuperscript{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 \textit{VHL} tumour suppressor gene and is characterised by predisposition to retinal and cerebellar haemangioblastomas, CCRC, and phaeochromocytoma.\textsuperscript{3,4} The lifetime risk of RCC in VHL disease is \textasciitilde70\% by the age of 60 years,\textsuperscript{5} but not all germline \textit{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).\textsuperscript{6} In addition, approximately 50\% of patients with isolated familial phaeochromocytoma have missense \textit{VHL} gene mutations,\textsuperscript{7} suggesting that specific \textit{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.\textsuperscript{8} These cases were characterised by dominant inheritance, early age at onset, and bilateral tumours. Although familial CCRC associated with chromosome 3 translocations was first described in 1979 (t(3;6) and t(3;8)),\textsuperscript{9,10} familial non-clear cell papillary RCC (HPRC) was not well defined until 1994.\textsuperscript{11,12} Subsequently, many cases of HPRC have been shown to have germline \textit{MET} gene mutations.\textsuperscript{13,14} 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 CCRC is limited. Recently, however, Teh \textit{et al}\textsuperscript{15} described two large kindreds with FCRC in which linkage to \textit{VHL} was excluded. A notable feature of these two kindreds was that, unlike CCRC in VHL disease, the diagnosis of RCC in these families was characterised by late onset (8/9 cases aged \textasciitilde50 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 CCRC 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

\textbf{Patients}
Families were referred by clinical geneticists and urologists. When possible, all cancer diagnoses were confirmed by tracing histopatho-
Familial clear cell renal cell carcinoma

Molecular genetic analysis

VHL gene

Mutation analysis was performed as described previously. 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.

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. A positive control germline 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 on our web site (http://www.bham.ac.uk/ICH/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).

CUL2 gene

The entire coding sequence of the CUL2 gene (exons 2 to 21) was screened for sequence variations as described previously. PCR conditions and primer sequences are available on our web site (http://www.bham.ac.uk/ICH/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-p26 (D3S1304, D3S1537, D3S1317, and D3S1038), 3p21-p24 (D3S1259, D3S2431, D3S2432, D3S2407, UBE1L, D3S2968, and D3S2407), and 3p14 (D3S1300 and D3S1481). UBE1L is a polymorphic 9 bp deletion at 3p21.2-p21.3. 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). 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-penetrance) 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...
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), 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 G2538 (exon 21, 3'UTR)).

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...
Familial clear cell renal cell carcinoma

containing a total of nine affected subjects, they found that eight out of nine cases were diagnosed >50 years of age and all had unilateral disease. These observations contrasted with younger age at onset of CCRCC in VHL disease and in chromosome 3p translocation families with CCRCC susceptibility. A young age at cancer diagnosis compared to sporadic cases is a feature of many inherited cancer susceptibility syndromes. In our series, we found that just over 50% of cases of FCRC were diagnosed <50 years of age, although the age at onset was, on average, not as young as that seen in VHL disease. The earliest reported age at diagnosis in our FCRC families was 21 years. The difference in age at onset of CCRCC in our FCRC kindreds and those reported by Teh et al might suggest heterogeneity in age at onset in FCRC families. In support of this contention, we note that the two largest families in our study either displayed an early age at onset (for example, family A) or a later age at onset (family E). One caveat in interpreting age at onset data would be the possibility that families with an early age at onset (particularly small families) will be preferentially ascertained. However, we note that if the age at onset analysis is restricted to those families with three or more cases of CCRCC in first degree relatives, 10 of 15 cases were diagnosed <50 years of age. Two of the families (kindreds H and I) included in our study consisted only of two first degree relatives with late onset (>50 years) CCRCC. Such familial clusters could be coincidental and may represent phenocopies rather than examples of genetic susceptibility to CCRCC. If so, exclusion of these families would have reduced the mean age of diagnosis of cancer in our FCRC families and would have changed the age distribution of RCC diagnosis in our FCRC families to resemble more that seen in VHL disease. The recognition that RCC diagnosis in FCRC families is often before the age of 50 years has important clinical implications. Morbidity and mortality from RCC in VHL disease has been reduced by regular surveillance and presymptomatic diagnosis of RCC in affected patients and at risk relatives. Such surveillance is also indicated in families in FCRC kindreds and we suggest that screening should be initiated from the age of 20 years (although this could be delayed in very extensive families with unequivocal evidence of a later age at onset). We did not find any evidence of susceptibility to non-renal cancers in FCRC kindreds. Although it is important to exclude VHL mutations in FCRC kindreds. The investigations performed would be expected to identify a germline VHL gene mutation in ~83% of VHL patients. Most mutations not detected by this approach are large, whole gene deletions (unpublished observations), which may be associated with more frequent haemangioblastomas than other types of VHL mutations. Although the chromosome 3p25 markers most closely linked to VHL (D3S1317 and D3S1038) were uninformative in our largest family, markers flanking VHL (D3S1537 and D3S1259) were not linked. Thus, our results in conjunction with those of Teh et al strongly suggest it is unlikely that germline VHL mutations account for a significant proportion of FCRC. Although MET gene mutation analysis has not been reported in FCRC previously, Teh et al excluded linkage to MET in the two FCRC kindreds they studied. It is possible that MET gene mutations in regions not covered by our analysis (for example, in the extracellular ligand binding domain) might occur in FCRC; however, analysis of sporadic papillary tumours and CCRCC have shown a tight correlation between genetic and histologic pathology. Thus, the molecular basis of CCRCC and papillary RCC appears to be distinct so that somatic VHL gene mutations are present in most sporadic CCRCC but not papillary RCC, and somatic MET mutations occur in papillary tumours and not clear cell tumours.

Chromosome 3p12-21 allele loss is frequent in sporadic CCRCC irrespective of whether the VHL gene is inactivated or not. This suggests that although VHL inactivation is a rate limiting step in the pathogenesis of most sporadic CCRCC, it is not sufficient and inactivation of a further chromosome 3p tumour suppressor gene(s) is necessary for CCRCC to develop. Hence, a chromosome 3p12-21 CCRCC TSG might represent a candidate gene for FCRC. However, available evidence from many tumour types suggests that chromosome 3p12-21 contains several tumour suppressor genes. For RCC, chromosome transfer studies have suggested RCC suppressor genes at both 3p12 and 3p21 and a constitutional t(3;8) translocation associated with a high risk of CCRCC maps to 3p14.2 close to the FRA3B fragile site and within the FHIT candidate TSG. Although FHIT has not been unequivocally implicated in sporadic CCRCC, it represented a plausible candidate for FCRC. However, we excluded linkage to various loci in 3p14-25 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 3p14-25 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.\textsuperscript{3, 35} 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.\textsuperscript{36-41} The ability of pVHL to bind Cul2 and elongin C appears to be important for pVHL function.\textsuperscript{37} 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.\textsuperscript{38} 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 (3;8) associated with CCRCC has been identified (TRC8) and shown to have some homology to the human patchel gene (PTC).\textsuperscript{42} Although the CCRCC susceptibility effect of chromosome 3 translocations may relate to chromosome instability rather than disruption of specific genes,\textsuperscript{43, 44} 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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